Reactive oxygen and nitrogen species in cystic fibrosis

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Award date:
2002

Awarding institution:
University of Bath

REACTIVE OXYGEN AND NITROGEN SPECIES IN CYSTIC FIBROSIS

Submitted by Mona Ratib Bustami

for the degree of PhD

of the University of Bath

2002

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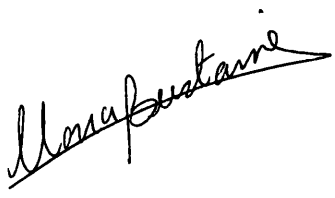

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To Ayman……..

ABSTRACT

Cystic fibrosis (CF) remains an incurable life limiting condition, which is the most common inherited lethal condition in Western countries. Despite the advances made in the general care of CF patients, modern research has been aimed at advances in the management of the respiratory infection, which is the main cause of the high mortality and morbidity rate among these patients. The normal respiratory epithelium serves as a dynamic barrier, participating in innate immunity and regulation of acquired immunity. Cystic fibrosis is a disease that primarily involves epithelia as a result of mutations of the cystic fibrosis transmembrane conductance regulator (CFTR), which is expressed in the apical epithelial membranes. Mutations in CFTR impair chloride transport and lead to an environment favoring colonization by pathogenic bacteria. In addition, there are other components that could lead to enhancement of bacterial infections. Respiratory inflammation and lack of nitric oxide are two important components that play a major role in the pathology of CF lung disease.

Using a pulmonary epithelial cell line, A549, the modulation of the production of nitric oxide (NO) was examined. These cells were found to produce NO with specific combinations of the pro-inflammatory cytokines interleukin (IL)-1β, interferon-γ (IFN), and tumour necrosis factor (TNF)-α. This NO generation was a result of activity of inducible nitric oxide synthase (iNOS). Modulation of this activity was achieved using the T cell-derived anti-inflammatory cytokine, IL-13, which significantly reduced NO generation. IL-13 was found to modulate iNOS activity in a PI 3-kinase dependent manner, and not via up-regulation of arginase.

Pulmonary colonization in CF patients with *Burkholderia cepacia* strains leads not only to a decline in respiratory function but also, in some cases, to acute systemic infection. Other patients exhibit persistent colonization with *B. cepacia* without deterioration of the respiratory system. Differences in catalase activities, that allow *B. cepacia* to resist oxidative killing, were observed from planktonic and biofilm cells and were consistent with susceptibility of strains to oxidative killing by H_2O_2. The biofilm cells had reduced catalase activity and were more susceptible to

oxidative killing. A fragment of the catalase gene of the clinical isolate *B. cepacia* J2315 strain was isolated, cloned and sequenced based on homology to the catalase (KatE) of *Escherichia coli* and *Pseudomonas aeruginosa*.

The two parts of this work aimed to highlight methods that may contribute to a better management of the respiratory tract infections in CF patients through the modulation of reactive nitrogen species and reactive oxygen species.

Acknowledgements

I have looked forward to typing this page as it makes the end of the work on my thesis and because so many people deserve thanks. First and foremost, I have to thank Dr. Anthony Smith for his awesome support and tremendous confidence. This thesis owes much to his tireless supervision. I am grateful to Dr. Malcolm Watson for his wise insights and advise. I would like to thank Prof. Tawfiq Arafat, Prof. Abdul Jalil Al-Thewani and Dr. Khalid M. for their support and constant encouragement. I am indebted to the University of Petra–Jordan, who funded this work and without whom it would not exist.

There are so many people who helped me to get through this, but I would like to acknowledge Dr. Karen W. for her friendship and support. A special mention goes to Dr. Sharmila, Dr. Charareh, Dr. Mick and Dr. Yuen they paved the way and taught me many of the techniques used here with patience and thorough skill. Also I would like to dedicate special thanks to all fellow PhD students for the company and friendship. But I would very much love to thank lots of friends who have made my staying in Bath most enjoyable, headed with Dina Al Mailam and her lovely family, I am in forever in their debt. I am also grateful for Rula A., Mona H., May A. and Fahima A. who shared me happy times as well as my parental guilt. I would like to dedicate very special thanks to Koumari A. who took care of my three daughters and myself tirelessly for the whole three years.

And finally, I could not have done it without the constant patience and understanding from my husband, Ayman, who has kept me in love, making sacrifices to allow me to pursue this goal. My daughters Nagham, Nadeen and Noor have provided me with a sense of reality, unwarranted unconditional admiration and lots of humour and hope during this process. Lots of thanks for my Mum and Dad, my brothers and my sister, for endless faith and encouragement.

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ABBREVIATIONS

ΔF508	Deletion of phenylalanine at residue 508
ABC	ATP-binding Cassette
ADP	Adenosine diphosphate
AHL	N-Acylhomoserine-lactone
ASL	Airway surface liquid
ATP	Adenosine triphosphate
AM	Alveolar macrophages
APS	Ammonium persulphate solution
BAL	Bronchoalveolar lavage
Bcc	*Burkholderia cepacia* complex
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine-5'-monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanisine-5'-monophosphate
Cl^-	Chloride ions
cNOS	Constitutive nitric oxide synthase
Da	Dalton
DAB	3,3' Diaminobenzidine
DAN	2,3-Diaminonaphthalene
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FBS	Foetal bovine serum

GTP	Guanosine triphosphate
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IκB	Inhibitor of κB
IPTG	Isopropyl β-D-thiogalactopyranoside
Kb	Kilo base
L-NMMA	N^{ω}-amino-L-arginine
LB	Luria Bertani
LPS	Lipopolysaccharide
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzyopyran-4-one
Mid-log	Mid logarithmic phase
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide binding domain
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase(s)
nNOS	Neuronal nitric oxide synthase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphatidyl inositol
PKA	phosphate protein kinase
PMN	Polymorphonuclear leukocyte
R	Regulatory domain
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
rpm	Revolutions per minute
STAT	Signal Transducer and activator of transcription
SEM	Standard error of the mean
SOD	Superoxide dismitase
TEMED	N,N,N',N'-tetramethylethylene diamine
Th 1	T helper 1
Th 2	T helper 2
TMD	transmembrane domain
TNF	Tumour necrosis factor
TBE	Tris-borate-EDTA buffer
TE	Tris.Cl-EDTA buffer
X-Gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside

Single letter amino acid codes

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln

R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

1. Introduction

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is the most common life-shortening genetic disorder in individuals of Northern Europe. It was in 1938 when Dr. Dorthea Anderson first coined the term "cystic fibrosis in pancreas" to describe pancreatic lesions associated with the disease. CF is a result of a recessive mutation in a single gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR). The gene product (CFTR) is expressed in the apical epithelial membranes, which makes it a disease that primarily involves epithelia. Mutations of the CFTR gene cause abnormal salt transport, particularly chloride (Cl^-), in the epithelia of the respiratory, hepatobilary, gastrointestinal, and reproductive tracts and the pancreas. The decrease in chloride transport is accompanied by decreased transport of sodium and water, resulting in dehydrated, viscous secretions that are associated with luminal obstruction and destruction and scarring of various exocrine glands (Welsh and Ramsey, 1998). As a result, the clinical manifestations of CF include pancreatic insufficiency in 80 to 90 percent of patients with a mutated CFTR gene, meconium ileus in 17 percent of affected newborns, diabetes mellitus associated with pancreatic disease in 20 percent of affected adults, obstructive biliary tract disease in 15 to 20 percent of all patients, and ozzospermia in more than 90 percent of affected men. The primary cause of morbidity and mortality in CF patients, however, is lung dysfunction, which accounts for over 90 percent of fatalities (Wood and Editor, 1996). In contrast to the lung, recurrent infection and inflammation is not a feature of CF in other organs. This indicates that there is a defect in local pulmonary defence mechanisms. Hypotheses

that explain the pathogenesis of CF lung disease need to correlate genetic mutation, inflammation, and infection. These are the cornerstones of understanding of CF lung infection.

1.2 The cystic fibrosis gene

The CF gene was identified in 1989. It is located on the long arm of chromosome 7, spans 250 kb of genomic DNA and contains 27 exons. The CF gene encodes a 1480-amino acid long transmembrane protein with asymmetrical structure (Riordan *et al.*, 1989; Rommens *et al.*, 1989; Kerem *et al.*, 1989). The protein was named cystic fibrosis conductance regulator (CFTR) reflecting the function of the protein, which is the regulation of chloride (Cl^-) channels (Collins, 1992) (Fig. 1.1). It is a multidomain protein subject to complex regulation. As a result, mutations in the CF gene can disrupt CFTR function by several different mechanisms according to their nature and where the mutation occurs (Vankeerberghen *et al.*, 2001). Accordingly, there are five main classes of mutations. Extensive population studies revealed that there are more than 1,000 different mutations (see CFTR mutation database at (http://www.genet.sickkids.on.ca/cftr/) (Pitt, 2001). The most common mutation among CF is a 3-bp deletion in exon 10 of the CF gene. This results in the loss of a single amino acid, phenylalanine at position 508 (ΔF508). CF mutations vary from nonsense, missense, frameshift and mRNA splice mutation. Most, but not all, of the mutations lead to severe pulmonary disease (Pier, 2000).

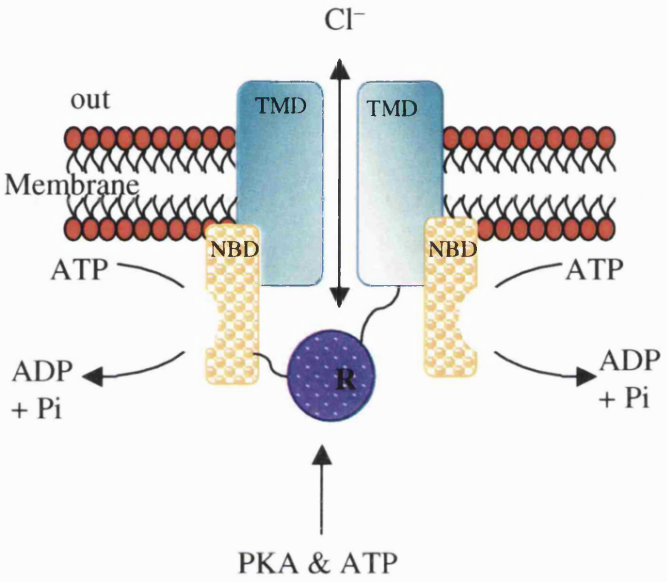


Fig. 1.1. Model showing the proposed domain structure of CFTR.. TMD, transmembrane domain; NBD,nucleotide-binding domain; R, regulatory domain; and PKA, cAMP-dependent protein kinase A.
Adapted from Welsh and Ramsey (1985).

1.2.1 The function of CFTR protein

Normal CFTR is an ATPase/ABC–transporter that functions as a channel for charged anions, such as chloride ions, to be selectively transported across the apical membrane of epithelial cells. There are two transmembrane domains (TMD) containing six transmembrane helices (TM), two nucleotide-binding domains (NBD) and a large hydrophilic regulatory (R) domain. Multiple kinases can activate the CFTR chloride channel, however, only protein kinase A (PKA) has been studied in detail (Vankeerberghen *et al.*, 2001). A simplified model of CFTR gating is given in (Fig. 1.2). It starts with phosphorylation of the R domain by cAMP-dependent PKA, which has a stimulatory role by enhancing the interaction of ATP with the nucleotide binding domains. These NBD hydrolyze ATP to control channel opening and closing (Cheng *et al.,* 1991). The channel opens and anions flow, according to the electrochemical gradient, through the pore formed by the TMDs. Once the R domain is dephosphorylated by phosphatases, the channel closes (Vankeerberghen *et al.*, 2001).

1.2.2 The secondary function of the CFTR

CFTR acts as a regulatory switch, since it regulates other ion channel proteins. It can negatively regulate the sodium (Na^+) transport by downregulating the amiloride sensitive epithelium sodium channel (ENaC) (Boucher *et al.*, 1986). As CFTR facilitates ATP release across membranes, it positively regulates the outwardly rectifying Cl^- channel (Deretic *et al.*, 1995).

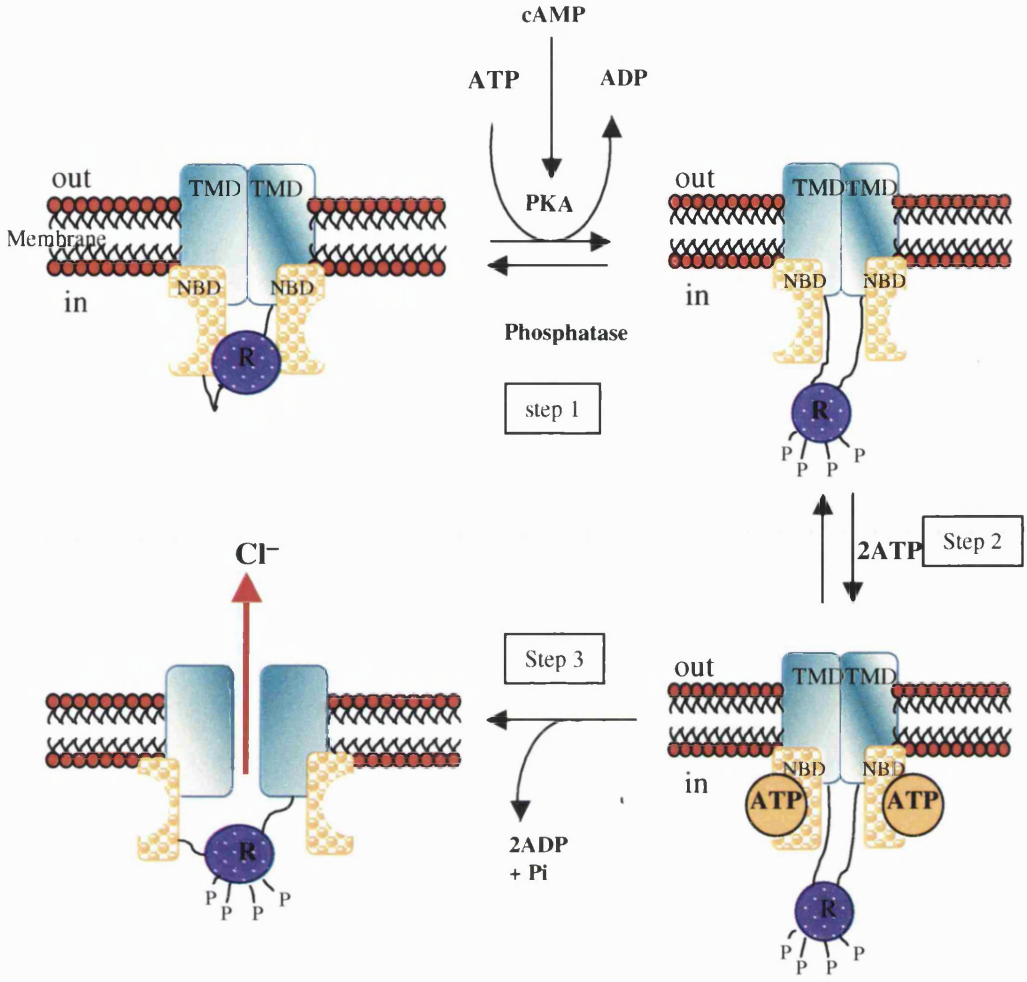


Fig. 1.2. A model of CFTR channel gating. Activation of the CFTR chloride channel starts after cyclic AMP stimulates PKA to phosphorylate the R domain (step 1), the CFTR channel is poised to bind ATP (step 2), which is cleaved to induce conformational charge (step 3), opening the chloride channel. Pi, inorganic phosphate; cAMP, cyclic AMP; PKA, protein kinase A; TMD, Transmembrane domain; NBD, nucleotide binding domain; R, regulatory domain and Cl^-, chloride ion. Adapted from Collins (1992).

1.2.3 Classes of CF gene defect

There are a variety of mechanisms by which mutations in the CF gene can result in quantitative or qualitative reductions in CFTR of CF patients. These mechanisms may be classified into five general categories, which are not mutually exclusive (Hilman and Constantinescu, 1999) (Fig. 1.3).

1- Class I mutations: These result in the formation of unstable/truncated CFTR mRNA, caused either by a base substitution that creates a premature stop codon, or by frameshift mutation. The resulting proteins either degrade rapidly or have little or no function. The disease phenotype of this class is often severe.

2-.Class II mutations: The majority of CF mutations, including the most frequent one ΔF508, are in this class. They lead to the production of an immature protein, due to defective processing or trafficking. The CFTR protein does not reach the apical membrane. The disease phenotype of this class is often severe.

3- Class III mutations: Interference with regulation of the chloride channel even though the protein reaches the apical membrane. It results in a decrease in the net chloride transport activity of the channel. This can also be a severe disease phenotype.

4- Class IV mutations: This class of mutations affects amino acids located in the pore of the channel. CFTR reaches the apical membrane but conduction is defective owing to altered channel properties such as gating. It often results in a mild disease phenotype.

5- Class V mutations: These lead to a decreased level of normal CFTR by mutations in the CF gene promoter. A CF phenotype may be produced if the reduction in CFTR is greater than 10%.

Patients with heterozygous mutations typically have the disease characteristics of the 'milder' mutation, due to the recessive nature of the disease (Vankeerberghen *et al.*, 2001)

1.3 Respiratory inflammation in cystic fibrosis

Respiratory inflammation is the most serious and common feature of early CF. Evidence suggest that this inflammation is not secondary to bacterial infection but rather supports the idea that dysregulation of the inflammatory response is an intrinsic component of the CF phenotype (Cantin, 1995). This report suggests a new model that links mutations of the CFTR and intracellular signal transduction, resulting in an exaggerated inflammatory response that is mediated, in part, through NF-κB. As a result, respiratory tract inflammation may occur prior to or in the absence of bacterial infection (Weber *et al.*, 2001). Armstrong and colleagues in 1997 found out that airway inflammation in infants and young children precedes respiratory infection. Their findings were supported by a longitudinal bronchoalveolar lavage (BAL) study of infants with CF who improved when pathogens are eradicated (Armstrong *et al.*, 1997). However, significant regional variability of lung inflammation in CF on BAL may suggest the possibility that Armstrong's findings may be insufficiently sensitive to detect infection (Meyer and Sharma, 1997). It is clear that in most studies made, not all elements of inflammation, infection and pulmonary function were studied

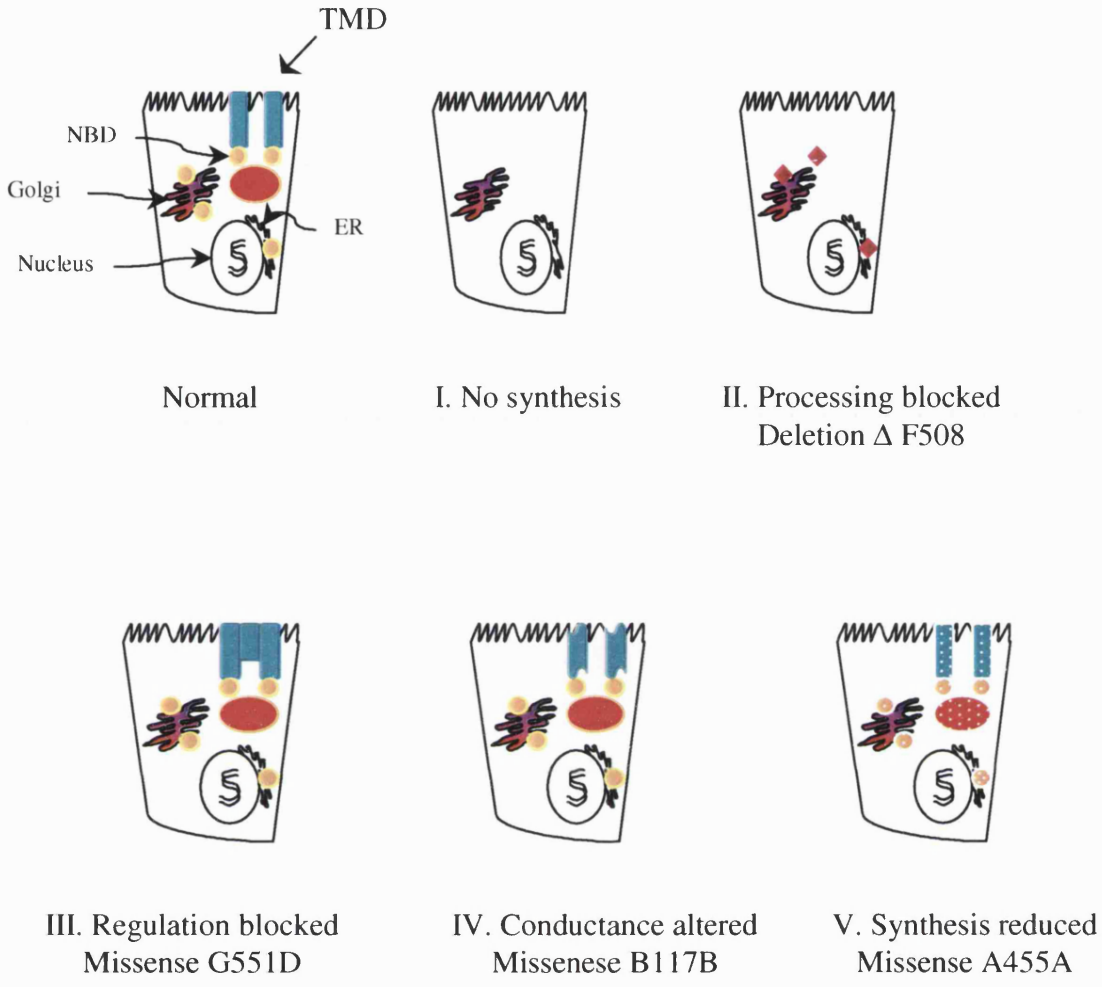


Fig.1.3. Classes of mutation affecting CFTR. Wild type *CFTR* is transcribed in the nucleus and processed in the Golgi and ER where it is inserted into vesicle membranes and transported to the apical epithelial cell surface to form a functional chloride channel. Five classes of mutations of cystic fibrosis transmembrane conductance regulator (CFTR) gene and proposed functions. The type of functional alterations of the chloride channel activity of CFTR due to each class mutation are illustrated. Class I, the mutations affect biosynthesis and no functional protein is synthesised ; Class II, mutations affect protein maturation and there are alteration in processing; Class III, mutations affect chloride channel regulation; Class IV, mutations affect chloride channel conductance; Class V. mutations cause reduced synthesis. TMD, transmembrane domain; MSD, nucleotide binding domain; R, regulatory domain; ER, endoplasmic Reticulum. Adapted from Hilman and Constantiescu (1999).

simultaneously in one group of infants, which would have given a better understanding of these relationships in early lung disease. In a recent study an attempt to investigate the effect of lower airway infection on clinical parameters, pulmonary function tests and inflammation in clinically stable infants and young children with CF, found a relationship between the bacterial lower respiratory tract infection and the presence of inflammation and abnormal pulmonary function (Dakin *et al.*, 2002). Cytokine analysis of bronchoalveolar lavage fluids (BALF) has revealed elevated levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-β) and IL-6 compared with healthy controls and other diseases in which there is bacterial infection in the lung (Bonfield *et al.*, 1995; Wilmott *et al.*, 1990). This increase in cytokine production in the CF airway is in part driven by lipopolysaccharide of pathogenic Gram-negative bacteria (Bonfield *et al.*, 1995). Moreover, observations concerning the role of respiratory inflammation in the development of CF respiratory disease indicate that epithelial cells recruit neutrophils locally through directed interleukin-8 (IL-8) secretion. Increased levels of IL-8, a pro-inflammatory chemokine that accounts for most of the neutrophil chemoattractant activity, have been shown from sputum and BALF of CF patients (Muhlebach. *et al.*, 1999; Armstrong *et al.*, 1997). On the other hand, some studies suggest that IL-8 production by CF respiratory epithelial cells is defective (Massengale *et al.*, 1999; Denning, *et al.*, 1998). Recently it has been demonstrated that defective CFTR function may result in increased NF-κB activation which may in turn result in increased pro-inflammatory cytokines secretion by airway epithelial cells (Blackwell *et al.*, 2001).

Interleukin-10 (IL-10) is an anti-inflammatory cytokine mediator constitutively produced by bronchial epithelial cells from normal individuals, that reduces production of TNF-α and IL-1β by macrophages, but is significantly absent in CF cells (Bonfield *et al.*, 1995). Thus, it might be predicted that absence of this cytokine causes the inflammatory response to be uncontrolled, resulting in increased intensity of lung injury (Massengale *et al.*, 1999; Bonfield *et al.*, 1995). A recent study demonstrated that immunoregulatory abnormalities caused by insufficient IL-10 not only affect the suppression of synthesis of pro-inflammatory cytokines, but would also alter the immunogenic function of macrophages (Soltys *et al.*, 2002).

1.3.1 The immune response and inflammation in CF respiratory tract

The primary function of the respiratory system is oxygenation of the blood and removal of carbon dioxide. This requires that air be brought into close proximity with blood. Accordingly, the respiratory tract is particularly exposed to infection either by microbes and airborne particles in inspired air or through the spread downward of bacterial cells commonly colonizing the nose and throat. As these agents are frequently deposited on the surface of the respiratory tract, efficient defence mechanisms are needed to avoid or minimise respiratory infections. The normal host defence of the respiratory system includes a rapid but non-specific reaction from components of the innate (non-specific) immune system (Zhang *et al.*, 2000).

More than 7000 liters of air mixed with inorganic and organic particles enter the respiratory tract, yet normal humans rarely develop pulmonary infections. However, infections of the CF pulmonary system, which is structurally normal *in utero* and

pathogen–free shortly after birth (Khan *et al.*, 1995) is the major cause of morbidity and accounts for more than 90% of the mortality in CF (Fitzsimmons, 1993).

1.4 Immunity of the respiratory tract

A first line of defence against pathogenic insult is called the innate immune system, which is followed by acquired immune responses associated with activation of T and B cells to specific antigens. However, the innate immune response not only provides the first-line barrier to colonization and infection but also determines to which antigens the acquired immune system responds and the nature of these responses (Fearon and Locksley, 1996) (Fig. 1.4).

1.4.1 Innate (non-specific) immune response

1.4.1.1 Physical defence

The initial line of defence for the respiratory tract is the barrier function of the ciliated epithelium. It effectively separates the luminal surface from the basolateral surface, via a mucus blanket which overlays a thin layer of airway surface liquid (ASL). Both mucus and ASL covers the surface of ciliated epithelia preventing bacterial adherence. Particles greater than 5μm in diameter are generally trapped in the mucociliary blanket. These mucus-trapped particles will be cleared by beating cilia, which propels the mucus up to the oropharynx for swallowing or expectoration (Zhang *et al.*, 2000). In the CF respiratory tract, defective CFTR protein reduces the secretions of Cl^- in the mucus layer and enhances Na^+ absorption, which reduces the water content of ASL. Matsui and colleagues supported this finding by presenting

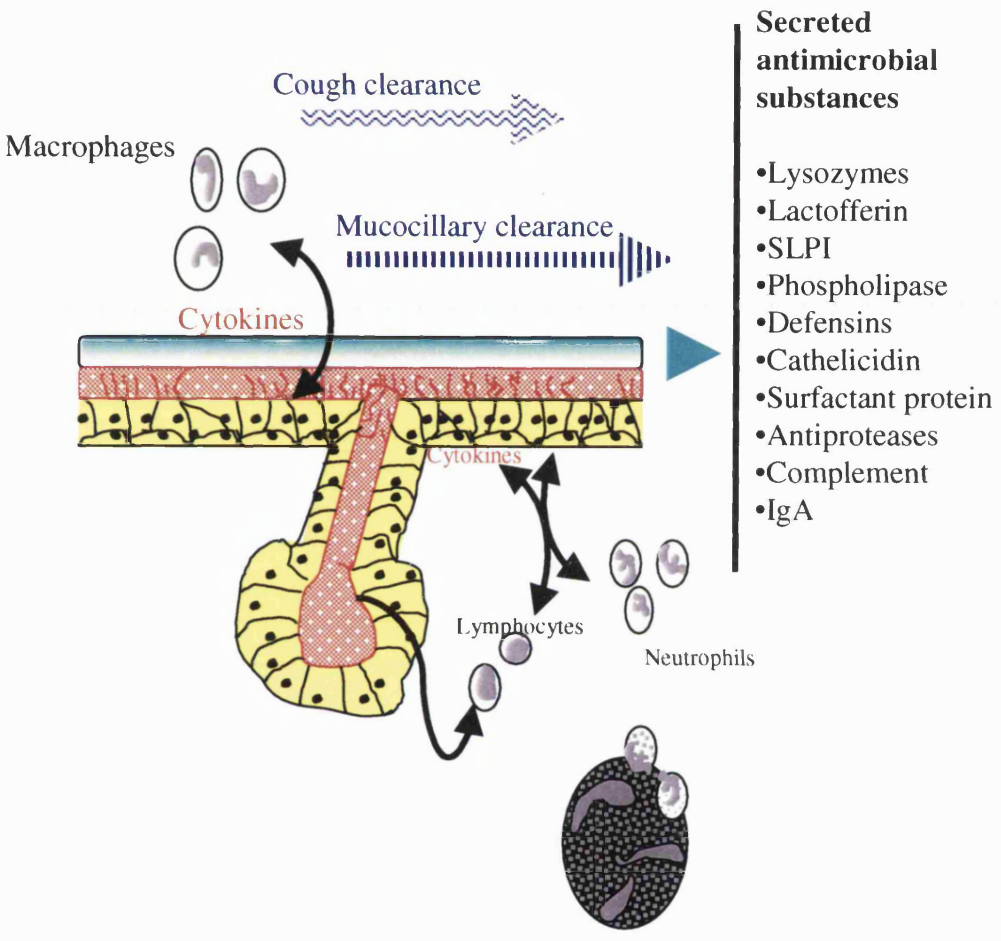


Fig. 1.4. A model of the host innate defence mechanisms of the respiratory epithelium. Cough and cilia mechanically remove inhaled debris and microorganisms entrapped in mucus, a mechanism called mucociliary clearance. Multiple substances with pro- and anti-inflammatory as well as antimicrobial activities are secreted by epithelial cells and function as innate immune barrier. Macrophages, B and T lymphocytes, neutrophils and also epithelial cells represent cellular component of the innate and adaptive immune system. Adapted from Bals *et al.* (1999).

evidence that CF epithelia hyperabsorb water (Matsui *et al.*, 1999). Thus, CF patients with infected respiratory tracts generally produce sputum that is dehydrated and highly viscous. Another hypothesis to explain how a defect in CFTR results in a breach of the innate immunity was proposed by Smith *et al.* (1996). They demonstrated that elevated salt concentrations in the ASL diminished the antibacterial activity of substances present in ASL *in vitro* assays, which consequently affect bacterial killing of CF ASL. Further analysis of the sputum indicates a high content of cellular and bacterial debris, such as: actin, serum, protein, DNA, alginate, and rigidifying lipids. These negatively charged biopolymers (mucin, DNA, and alginate) form a viscoelastic network, which reduces the efficiency of the diffusion barrier for pathogens and harmful particles (Sanders *et al.*, 2000), and reduces the ciliary beat frequency. This consequently impairs clearance of the mucus blanket.

1.4.1.2 Chemical defence

Particles less than 5μm in diameter bypass the mucus barrier and may gain access to the terminal respiratory tract. To insure sterility of all parts of the respiratory tract, a wide range of peptides, proteins and organic molecules derived from secretory cells are found in the epithelia. These secretory cells include mucous and serous cells in submucosal glands, as well as goblet and clara cells in the epithelial layer. These substances either exert direct lethal activity or facilitate the elimination of infectious pathogens by phagocytosis. These include lysozyme, complement, immunoglobulin A and G, fibronectin, lactoferrin, transferrin, phospholipase A2, lipopolysaccharide (LPS)-binding protein, β-defensins, cathelicidins, collectins, leukoprotease inhibitor,

peroxidase, reactive nitrogen species (RNS) and nitric oxide (NO) (Zhang *et al.*, 2000; Diamond *et al.*, 2000). In the normal respiratory tract the activity of some of these chemical is salt concentration-dependent. In CF the bactericidal activity of ASL was reduced as a result of the high NaCl concentration. For example, the low molecular weight, heat stable defensin molecule has a broad-spectrum antibacterial activity that is dependent on salt concentration (Bals *et al.*, 1999). This salt-dependent inactivation of defensin has been proposed to play an important role in the pathogenesis of CF (Smith *et al.*, 1996).

As part of the respiratory tract response to infection, two complementary inducible defence mechanisms have been observed. The first mechanism is the increased production of antimicrobial agents, and the second mechanism is the induction of a signaling network to recruit phagocytic cells to contain the infection. Constitutive peptides and proteins are up-regulated in response to inflammatory mediators, pro-inflammatory cytokines, like IL-1β, TNF-α, IFN-γ, Gram-negative lipopolysaccharide (LPS), Gram-positive lipoteichoic acids, lipoarabinomannans of myobacteria, and bacterial-derived DNA and lipoproteins. Human β-defensin showed increased levels in inflamed respiratory cells in response to *P. aeruginosa* LPS, IL-1β, as well as TNF-α (Diamond *et al.*, 1996; Russell *et al.*, 1996). Other inducible host defence molecules include mucin and reactive nitrogen species such as nitric oxide (NO). Mucin levels are elevated in the BALF in response to Gram-negative and Gram-positive infections. Moreover, *P. aeruginosa* LPS induces mucin glycoprotein synthesis via upregulated *MUC2* and *MUC5A* genes in the lung (Diamond *et al.*, 2000).

The production of reactive nitrogen species (RNS) in mammalian hosts appears to play a role either solely or in combination with other radicals in the defence against respiratory tract infection (Nathan and Hibbs, 1991; Nathan, 1992; Fang, 1997; Nathan and Shiloh, 2000). The increasing susceptibility of CF patients to persistent bacterial infections has been attributed, in part, to the low availability of RNS, and nitric oxide (NO) in particular (Kelley and Drumm, 1998; Meng *et al.*, 1998). The following section will discuss the role of NO in immune response.

1.5 Nitric oxide and Reactive nitrogen species

In healthy human subjects, nitric oxide (NO) can be measured in exhaled breath. NO levels increase significantly in several lung diseases, notably asthma and bronchiectasis (Hamid *et al.*, 1993), or after exposure to irritant gases such as ozone (Pendino *et al.*, 1993). Nitric oxide is synthesised via constitutive or inducible forms of nitric oxide synthases (NOSs) (Section 1.5.1). The oxidative metabolism of NO involves a number of reactive nitrogen species (RNS) in which the oxidation state of nitrogen ranges from +1 to +5 (Table 1.1). Several of these species exhibit the various biological actions of NO. In human, RNS are released from several cell types within the respiratory tract, including airway and alveolar epithelial cells, macrophages, neutrophils, mast cells, and vascular endothelial and smooth-muscle cells. Production of NO is known to be constitutive in all areas of the healthy respiratory tract (van der Vliet *et al.*, 1999).

Symbol	Name	Oxidation state
NO^-	Nitroxyl anion	+1
N_2O	Nitrous oxide	+1
NO	Nitrogen monoxide	+2
NO^+	Nitrosyl (nitrosonium) cation	+3
NO_2^-	Nitrite	+3
N_2O_3	Dinitrogen trioxide	+3
NO_2	Nitrogen dioxide	+4
N_2O_4	Dinitrogen tetraoxide	+4
$ONOO^-$	Poxynitrite	+5
NO_2^+	Nitryl (nitronium) cation	+5
NO_3^-	Nitrate	+5

Table 1.1. Types of biological reactive nitrogen species (RNS). Adapted from va der Vliet *et al.* (1999).

1.5.1 NO: Chemical and biochemical basis of production

Nitric oxide is a radical gas, which has diverse biological functions in the cardiovascular, nervous and immune systems. NO is lipid and water-soluble and has a molecular weigh of 30-dalton with an apparent half-life between 6 and 60 seconds. It is synthesized from the amino acid L-arginine, by a family of enzymes called nitric oxide synthases (NOSs). There are three mammalian isoforms of NOS that catalyze the production of NO by the same biochemical pathway, which consists of two sequential monooxygenase reactions (Fig. 1.5). One molecule of L-arginine is oxidized at a guanidine nitrogen to produce N^{ω}-OH-L-arginine as an intermediate, which is further oxidized to yield one molecule each of NO and L-citrulline. This oxidation reaction needs NADPH and dioxygen as cosubstrates for NOS (Nathan, 1992).

The three distinct human isoforms of NOS are products of three different genes, which share 51-57% homology. They differ in their localization, regulation, catalytic properties and inhibitor sensitivity. They can be divided into two main groups characterized broadly as the constitutive (cNOS) and inducible (iNOS) isoforms. The constitutive NOS isoforms are usually referred to as: neuronal NOS (nNOS, also known as Type I, NOS-I and NOS-1) being the isoform first found (and predominating) in neuronal tissue. Endothelial NOS (eNOS, also known as Type III, or NOS-III and NOS-3) is the isoform found in vascular endothelial cells. The third isoform is inducible NOS (iNOS), also known as type II, NOS-II and NOS2), which is inducible in a wide range of cells and tissues. The nNOS and eNOS are exquisitely sensitive to calcium, and/or calmodulin levels, while the iNOS does not require either

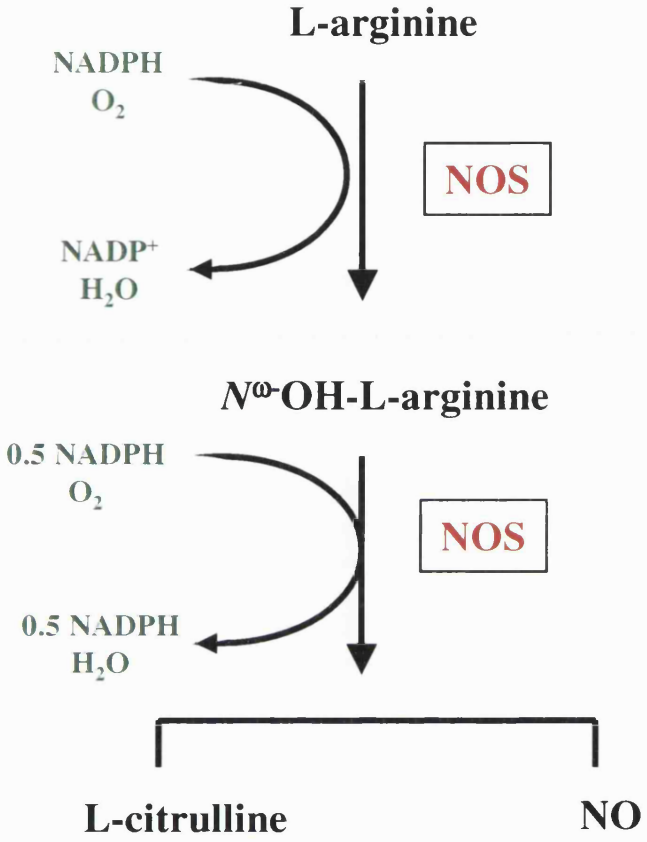


Fig. 1.5. Biochemical pathway of NO production in mammalian cells. Two sequential monooxygenase reactions are catalyzed by NO synthase (NOS).

for its activity. The subunit molecular mass of nNOS ≈ 160 KDa, while eNOS and iNOS ≈130KDa (Knowles and Moncada, 1994; Stuehr, 1999).

It has been demonstrated that iNOS is induced by pro-inflammatory cytokines, viral infection and microbes or microbial products such as lipopolysaccharide of Gram-negative bacteria (Hoffman, 1996). Interestingly, variable immunostaining of airway epithelium revealed that iNOS is constitutively expressed in airway epithelial cells of normal, non-smoking individuals (Guo *et al.*, 1995).

Once formed, NO diffuses to adjacent cells where it activates soluble guanylate cyclase, resulting in the formation of cyclic guanosine monophosphate (cGMP), which in turn mediates many, but not all, of the biological effects of NO. The specific activation of guanylate cyclase by NO is due to its ability to bind transition metal iron heme (Moncada and Higgs, 1993). Nitric oxide is relatively inocuous, unless it couples with other radical species, such as superoxide (O_2^-). This property allows it to function as a physiological messenger molecule and, under certain conditions, as a cytotoxic effecter molecule as well (Gross and Wolin, 1995).

In vivo NO can be detected as gas, which can react with O_2 to form nitrite (NO_2^-), nitrate (NO_3^-), and nitroxyl (NO^-) and intermediates such as nitrosonium (NO^+), peroxynitrite ($ONOO^-$) and nitrogen dioxide ($\cdot NO_2$). Reaction of NO with hydrogen peroxide produces an singlet oxygen molecule, which is potentially toxic (Morcillo *et al.*, 1999) (Fig. 1.7). It is noteworthy that NO is not necessarily the first RNS intermediate formed. Nitric oxide synthesis is often secondary to the buildup of nitrite and nitrate, or from reactions with haem proteins, such as the oxidation of

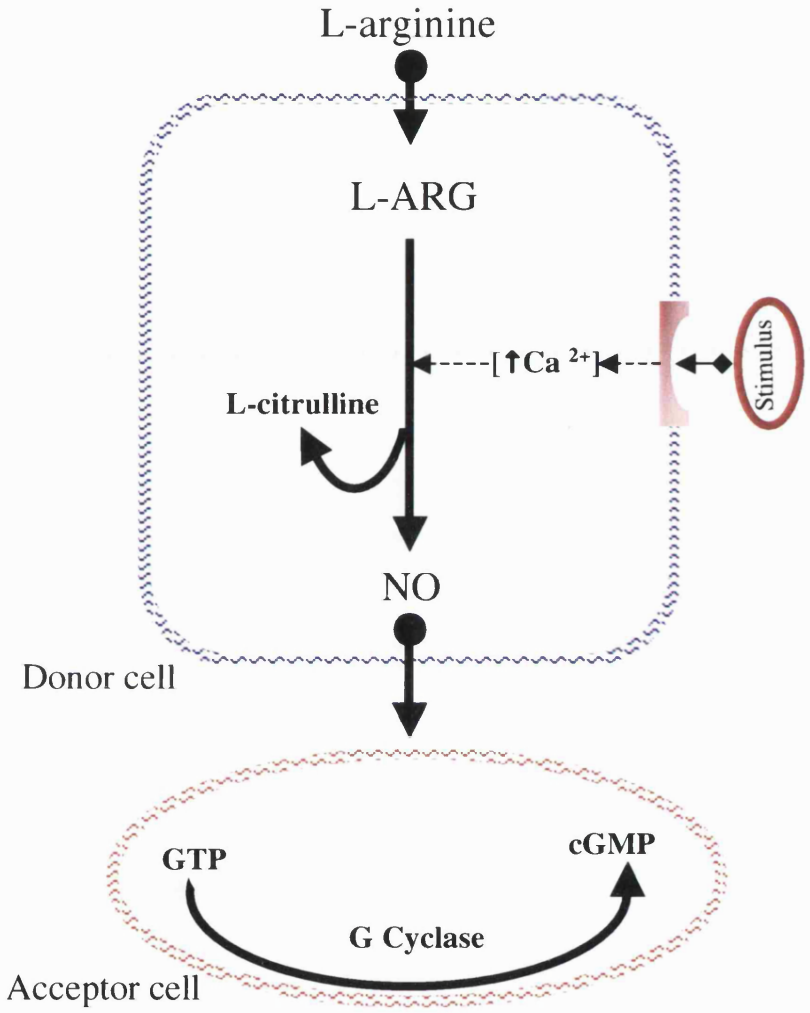


Fig. 1.6. Biosynthesis of nitric oxide (NO) from L-arginine (L-ARG). Release of NO activates guanylate cyclase in an acceptor cell leading to elevation of intracellular cyclic GMP (cGMP) levels. Adapted from Whittle (1995).

oxyhaemoglobin to methaemoglobin, or the stimulation of guanylate cyclase. Accordingly, other potential RNS reactions may lead to the formation of NO (Alderton *et al.*, 2001). Thus, NOSs are not the only source of NO in exhaled air and as such its measurement may not be a direct measure of NOS activity in the lower respiratory tract.

Nitric oxide reacts with thiol-containing molecules such as cystein and glutathione to form S-nitroso proteins and S-nitrosothiol. S-nitrosothiol may function as a reservoir, or storage pool, for enzymatically synthesized NO. It was reported that 70 to 90% of NO is released from S-nitrosothiols, which are potent relaxants of the human airway. They may play an important role in sequestration, release, and transportation of NO to its site of action. In addition, NO gas may be released from nitrite protonation to form a nitrous acid in an acidic environment. This pH-related pathway has been implicated in asthma (Kharitonov and Barnes, 2001).

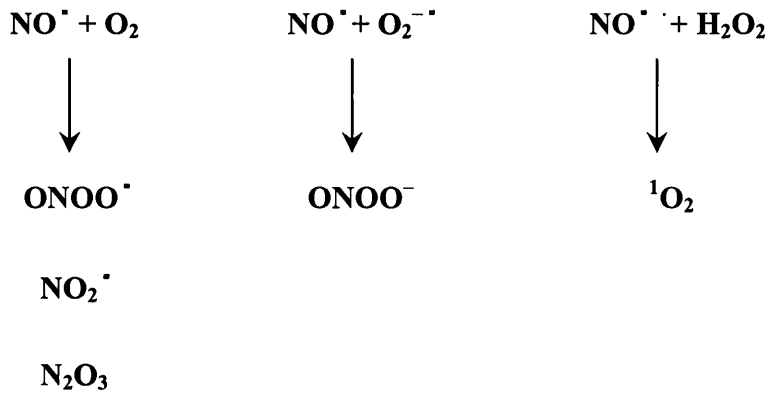


Fig. 1.7. Reaction products of NO with oxygen or reactive oxygen species.

1.5.2 Biological activity of NO

The activity of NO has been described as a double-edged sword, serving as a regulatory and/or potent anti-inflammatory molecule, as well as a toxic and/or pro-inflammatory molecule. The reason behind this contradictory property of NO is not entirely clear. However, the chemistry of NO and its metabolites may help identify which of the many NO-dependent reactions are important in modulating the inflammatory response (Grisham *et al.*, 1999) (Fig. 1.8).

The biological actions of NO appear to be mediated by its ability to interact with paramagnetic centres in effector proteins, such as heme- or iron-sulfur centres. On the other hand, RNS are reactive toward all classes of biomolecules, such as transition-metal ions in metaleproteins, amino acid residues (thiols, amines, and tyrosine), unsaturated lipids, and DNA bases. Such interactions represents the cytotoxic and cytostatic processes associated with excessive NO production (van der Vliet *et al.*, 1999; Poole and Hughes, 2000).

In another attempt to classify NO physiological properties Grisham and colleagues (Grisham *et al.*, 1999) considered the timing, location and the rate of production as a scheme to divide it into direct and an indirect effects (Fig. 1.9). Direct effects are those reactions in which NO interacts directly with a biological molecule or cellular target and are thought to occur under normal physiological conditions when NO fluxes are low. Indirect effects are those reactions mediated by NO-derived species

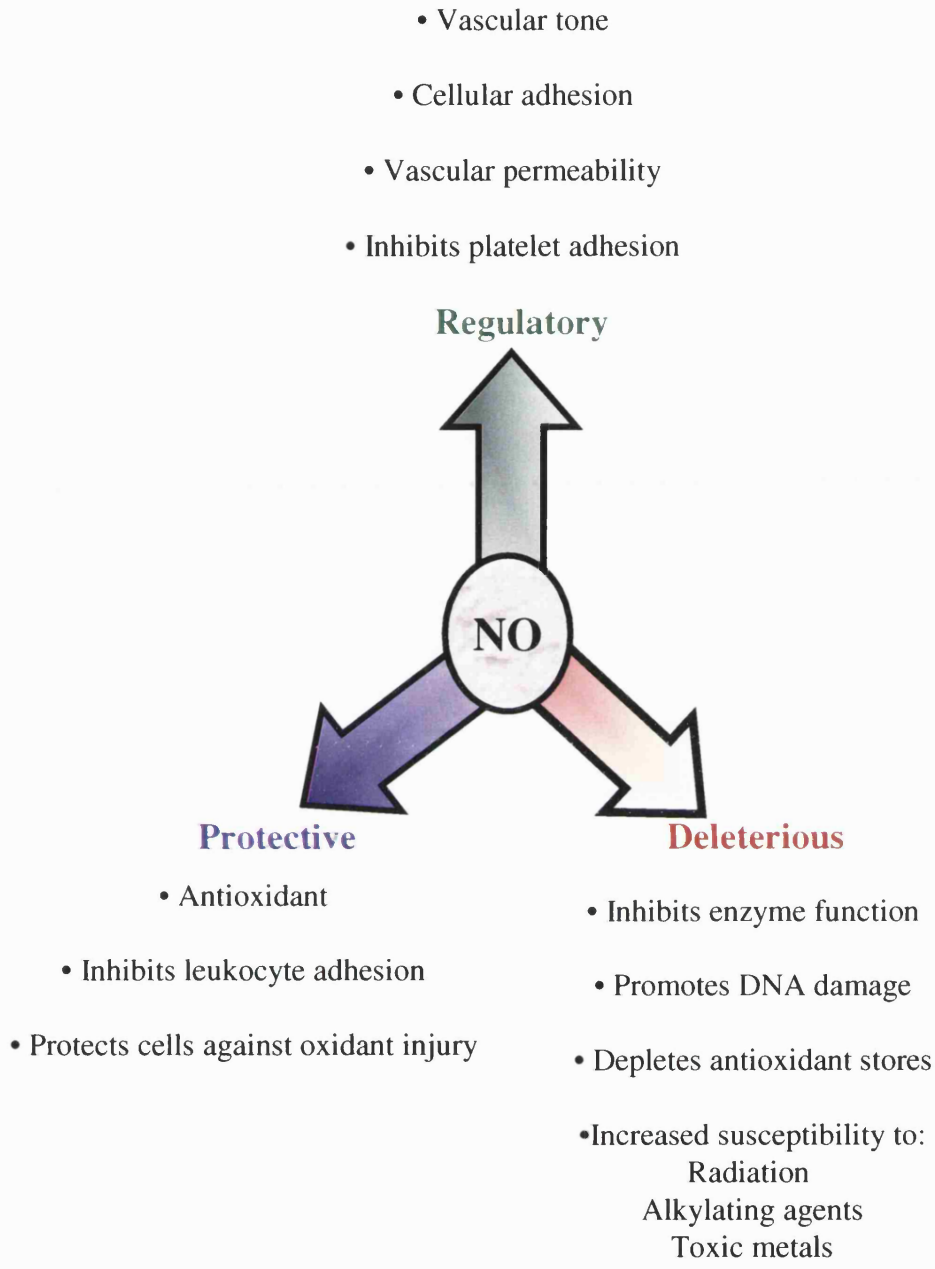


Fig. 1.8. Regulatory, protective and deleterious biological effects of nitric oxide (NO).

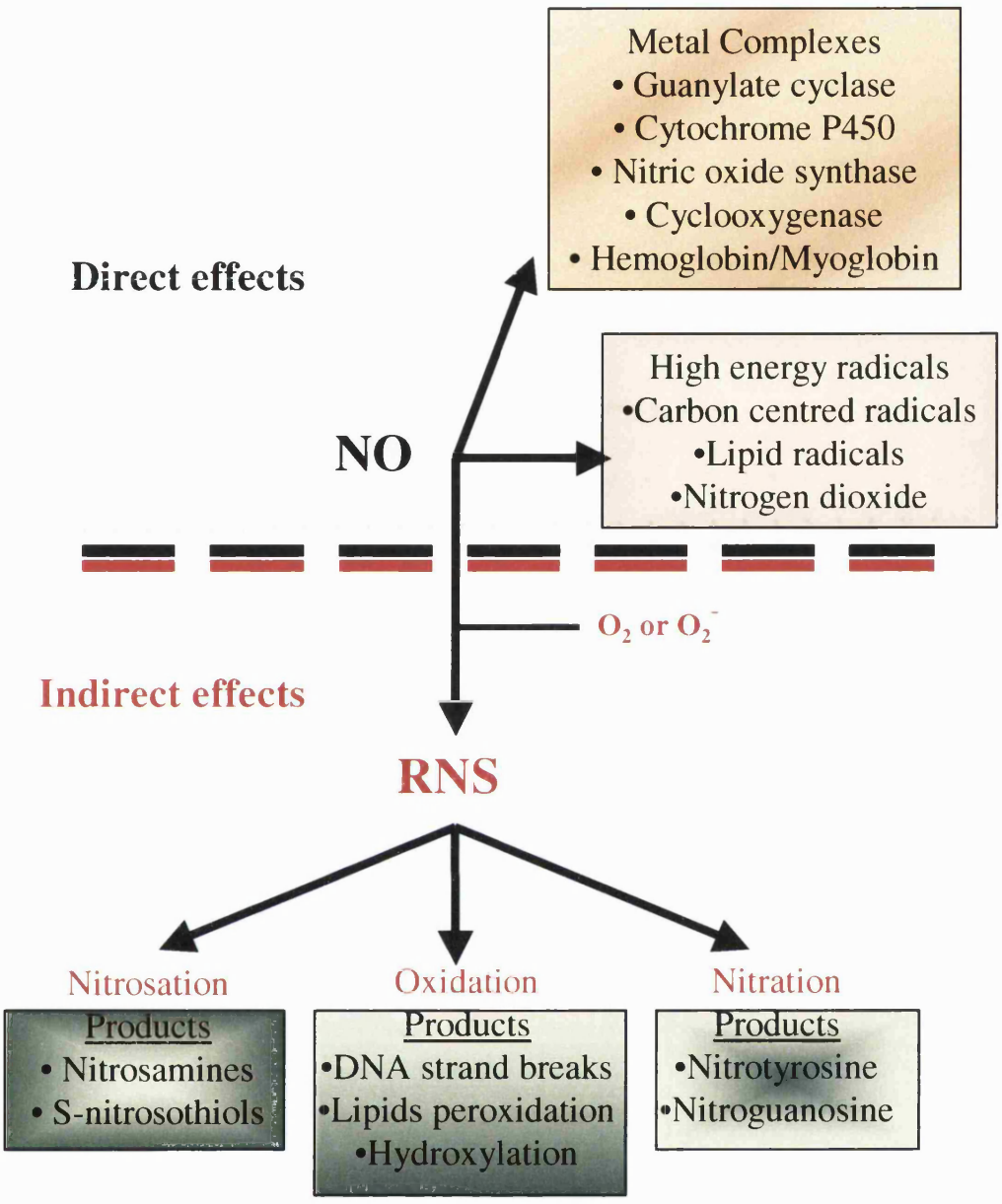


Fig. 1.9. Physiological chemistry of NO. RNS, reactive nitrogen species. Adapted from Grisham *et al.* (1999).

such as reactive nitrogen oxide species resulting from the reaction of NO with oxygen or superoxide and are produced when fluxes of NO is high. In general the direct types of reactions may be represented as regulatory and/or anti-inflammatory, and the indirect types prevail during active inflammation. Thus, to determine the toxic or protective roles of NO in biological systems one should consider the relative rates of its production, its location, rates of reaction with reactive oxygen species and the presence of antioxidants and tissue mediators (Grisham *et al.*, 1999; Freeman *et al.*, 1995)

1.5.3 Reactive nitrogen species in immunology

The antimicrobial functions of NO have been evident from the inhibitory role of macrophage iNOS-derived NO to invading microbes. Macrophage-derived RNS are likely to inhibit microbes in a manner that is similar to the mechanisms by which RNS inhibit mammalian target cells. Reactive nitrogen species have been shown to modify DNA, proteins, and lipids, as well as exert indirect effect on microbes by modulating immune responses or other host cell functions (Nathan and Hibbs, 1991; Brunelli *et al.*, 1995; Nathan and Shiloh, 2000).

Secondary reactions of reactive oxygen species (ROS), which are produced by all aerobic cells, with RNS potentiate the antimicrobial reactivity of RNS (Zhu *et al.*, 1992). Since their interaction results in the production of a variety of antimicrobial molecular species, such as, peroxynitrite $ONOO^-$, which is a potent oxidant as well as nitrating and hydroxylating agent. Peroxynitrite have greater cytotoxic potential than

NO or O_2 alone (Fang, 1997). However, RNS cannot discriminate the genomic source of their chemical targets.

Reactive nitrogen species are critical in host defence not only because they can inhibit pathogens, but also because they are immunoregulatory. They can inhibit G proteins, activate or inhibit kinases, caspases, metalloproteases, transcription factors, and DNA methyltransferase, inhibit lymphocyte proliferation, alter cytokine and prostaglandin production, and either cause or prevent apoptosis of host cells (Nathan and Shiloh, 2000).

1.5.4 Modulation of nitric oxide synthase activity

The iNOS gene is predominantly regulated at the level of transcription by synergistic combinations of pro-inflammatory cytokines and bacterial cell wall product notably LPS (Xie and Nathan, 1994; Robbins *et al.*, 1994; Tayler *et al.*, 1998). Sequence analysis of the 5'-flanking region of iNOS promoter revealed consensus sequences that are implicated in cytokine-modulated gene expression, namely, transcriptional factor, nuclear factor- κB (NF-κB).

NF- κB is a heterodimer of proteins of the c-REL family of transcription factors which are constitutively expressed and retained in the cytoplasm, associated with an inhibitory protein known as inhibitory κB (IκB). Activated NF- κB complexes are translocated to the nucleus in response to mitogens, cytokines and LPS of Gram-negative bacteria. Activation of NF- κB requires phosphorylation and degradation of IκB proteins (Casado *et al.*, 1997) (Fig. 1.10). NF-κB is important in the induction of

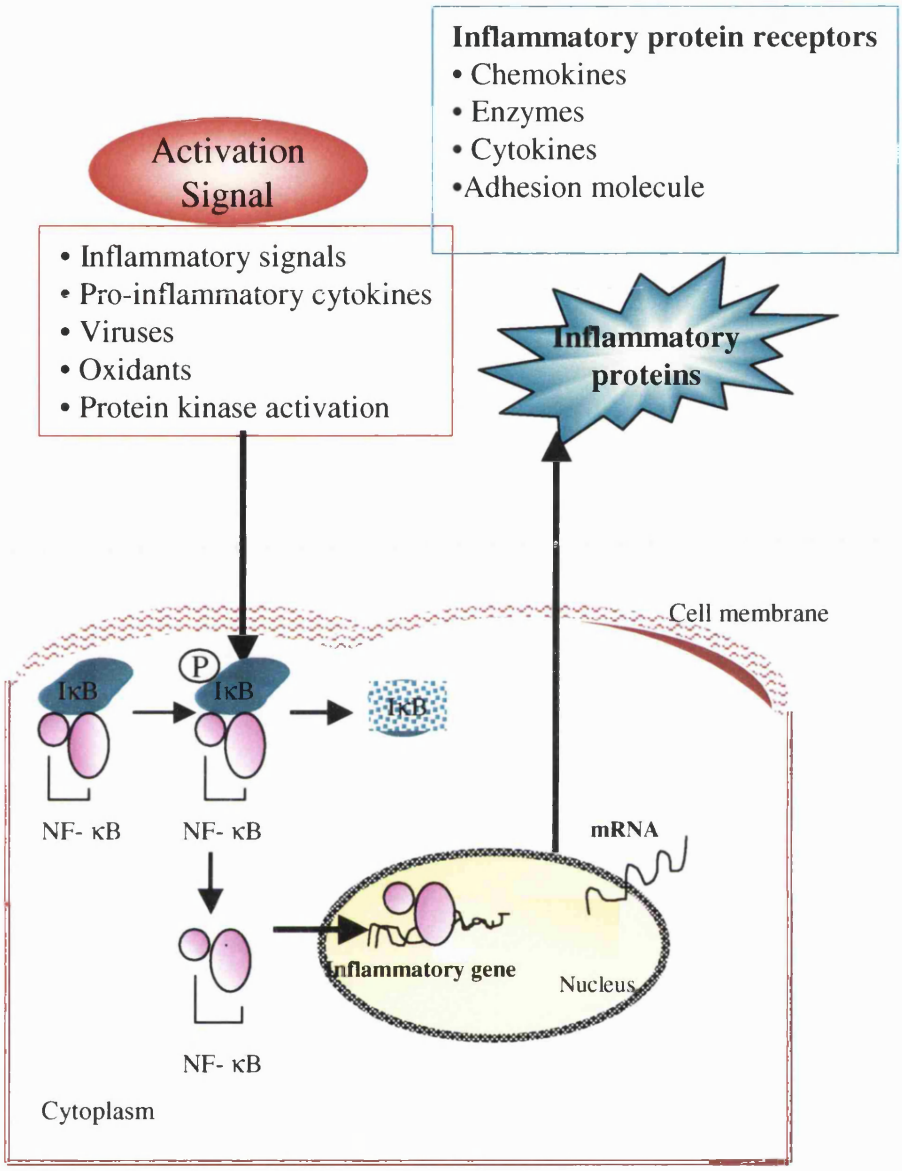


Fig. 1.10. Schematic diagram of nuclear factor -κB (NF- κB) leading to gene transcription in lung epithelial cells. Activation of NF- κB involves the phosphorylation, ubiquitination and subsequent proteolytic degradation of the inhibitor of NF- κB (IκB). Free NF- κB then translocates into the nucleus and binds to its consensus sites. The activation of NF- κB leads to the co-ordinate expression pro-inflammatory genes. Adapted from Barnes and Karin (1997).

murine iNOS, whereas, it appears to be less important in the regulation of human gene (Chu *et al.*, 1998). Human iNOS regulation appears to require IFN-γ and the activation of the NF-κB and activators of transcription (STAT)-1 (Guo *et al.*, 1997)

Interleukin (IL)-13 and the related cytokines IL-4 and IL-10 are regulatory cytokines secreted by activated T helper (Th) 2 cells. They have been proposed to induce variety of immunomodulatory functions on a wide range of cell types, including macrophages, natural killer (NK) cells, fibroblasts, airway smooth muscle cells, endothelial cells and eosinophils (Brombacher, 2000). Interleukin-13 has been shown to display anti-inflammatory properties that include modulation of monocyte and B cell function, suppression of cytotoxicity mediated by monocyte production of pro-inflammatory cytokines including TNF-α, IL-1α and IL-1β, IL-6, IL-8, and macrophage inflammatory protein (MIP)-1α (Zurawski and Dervies, 1994). IL-13 can modulate IL-8 and inhibit iNOS and cyclooxygenase-2 (COX-2) expression in the human colon epithelial cell line, HT-29 (Kolios *et al.*, 1996 and *et al.*, 1998). In the lung, IL-13 and IL-10, but not IL-4, were identified as endogenous cytokines that exhibit an anti-inflammatory effect via the suppression of NF-κB during an acute inflammatory lung injury. Inhibiting the endogenous IL-13 and IL-10 with antibodies enhanced NF-κB activation and caused an increase in intrapulmonary production of TNF-α, which was associated with increased accumulation of neutrophils and increased leakage of albumin (Lentsch *et al.*, 1999).

Several groups have shown that during pro-inflammatory cytokine or LPS treatment, IL-4, IL-10 and IL-13 acted as negative modulator of NO synthesis. However, their inhibitory effects are tissue specific (Saura *et al.*, 1996; Wright *et al.*, 1997; Diaz-

Guerra *et al.*, 1999; Rutschman *et al.*, 2001). In the human pulmonary epithelial cell line A549 and the human colon epithelial cell line HT-29, IL-13 and IL-4, but not IL-10, inhibited transcriptional iNOS up-regulation by pro-inflammatory cytokines (Berkman *et al.*, 1996; Wright *et al.*, 1997). Moreover, the inhibitory pathways of iNOS in activated HT-29 cells, renal mesangial cells and murine macrophages were via up-regulation of IκB levels and this effect was attributed to the action of phosphatidylinositol 3-kinase (Donaldson *et al.*, 1996; Wright *et al.*, 1997; Diaz-Guerra *et al.*, 1999)

1.5.5 Nitric oxide synthases and CF

Despite the inflammatory nature of CF lung disease, levels of exhaled NO have been reported as either normal or low when compared with healthy non-smoking controls (Kelley and Drumm, 1998). Grasemann *et al.*, (1997) observed a significant reduction in levels of NO from CF patients when compared with nonsmoking controls. Moreover, another study showed that exhaled and nasal NO is reduced in adult patients with CF, and there was no correlation between exhaled and nasal NO and genotype, or with infection status (Thomas *et al.*, 2000). The reason behind decreased concentrations of expired NO in CF is still controversial. Kelley and Drumm (1998) demonstrated a lack of iNOS expression in murine CF respiratory epithelium, and CF human trachea. They also correlated low activity of iNOS to sodium hyperabsorption and susceptibility to bacterial infection. More recent studies have hypothesized that nNOS makes an important contribution to expired NO. Animals with non-functional *nNOS* gene had 40% less NO compared with wild-type mice (De Sanctis *et al.*, 1997).

Neuronal NOS-derived NO is the main neurotransmitter of human nonadrenergic, noncholinergic (NANC) nerves that are involved in human bronchomotor control. It was found that the inducible NANC system is abnormal in CF, which may lead to airway obstruction and alteration of pulmonary functions (Belvisi *et al.*, 1995).

Measuring NO levels in expired air may not be a useful clinical indicator of CF. Since sputum NO_2 /NO_3 analysis demonstrated an elevated concentration from CF patient with acute pulmonary exacerbation compared with stable CF patients and normal control objects. This finding suggests that NOS is activated in CF with acute exacerbations and NO is trapped in the airway secretions (Linnane *et al.*, 1998). Other studies explained the low NO that it combines with superoxide anion produced by neutrophils to produce peroxynitrite that can form nitrotyrosine or decompose to nitrate (Jones *et al.*, 1998; Robbins *et al.*, 2000).

1.6 Cell mediated immunity of the respiratory tract

1.6.1 Alveolar macrophages

Alveolar macrophages (AM) constitute the first line of phagocytic defence against infectious agents that evade the mechanical defences. They lie on the alveolar walls and gain access to the gas-exchanging airways. These cells have important phagocytic, microbicidal, and secretory functions that are essential to lung immunity via initiating inflammatory and immune responses. Alveolar macrophages phagocytose microbial challengers to maintain sterility of the alveolar space, which lack mucociliary properties. Alveolar macrophages are capable of generating numerous mediators that orchestrate the recruitment of large numbers of neurophils, or polymorphonuclear

leukocytes (PMN), from the pulmonary vasculature into the alveolar space. The recruitment of neutrophils supports the overall phagocytic capacity leading to complete eradication of invaders (Zhang *et al.*, 2000). Alveolar macrophages encounter multiple chemoattractant signals that can potentially guide their path. These AM-derived substances include complement, arachidonic acid metabolites such as leukotriene B4, chemotactic peptides such as IL-8, and macrophage inflammatory protein (MIP)-2, and other related chemokines (Sanchez-Madrid and Del Pozo, 1999). In CF it is increasingly recognized that neutrophils-dominate the lower respiratory tract, and yet bacteria are not eradicated. Accordingly, more active neutrophils are recruited but efficient phagocytosis and lysis of *P. aeruginosa* still does not take place. High concentrations of elastase are released from the short-lived phagocytosis which inactivate opsonins and their cognate receptors on the surface of viable phagocytosis, by proteolysis. This may lead into a repetitive vicious cycle of fooled self-destructive host-defence which causes an extensive brochiectasis, destructive emphysema and fibrosis of pulmonary tissue (Tummler and Kiewitz, 1999).

1.6.1.1 Production of reactive oxygen species

At the onset of phagocytosis stimulated PMLs and AMs undergo what is termed respiratory burst or oxidative burst. It is a coordinated sequence of biochemical reactions characterized by an accelerated O_2 consumption to correspond to the high rate of oxidative phosphorylation, followed by the one-electron reduction of oxygen to superoxide anion ($O_2^{\bullet -}$) using NADPH or NADH as the electron donor and catalysed by a NAD(P)H oxidase (Fig. 1.11).

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2^{\bullet -}$$

Superoxide anion ($O_2^{\bullet -}$) is relatively unstable, with a half-life of only milliseconds. Because it is charged it does not cross the membrane vesicle that contains the engulfed pathogen. However, it will react with proteins that contain transition metal prosthetic groups, such as haem moieties or iron-sulfur clusters leading to protein damage or protein/enzyme inactivation. Most of the $O_2^{\bullet -}$generated *in vivo* undergoes a nonenzymatic or superoxide dismutase (SOD)-catalyzed reaction resulting in hydrogen peroxide (H_2O_2) (Repine *et al.*, 1997).

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide (H_2O_2) can be oxidized by neutrophil–specific peroxidase (MPO), as well as eosinophil-specific peroxidase (EPO) in the presence of a halide cosubstrate, to form a potent oxidant hypohalous acid (HOX) and other reactive halogenated species.

$$H_2O_2 + X^{\bullet -} + H^+ \longrightarrow HOX + H_2O$$

Much of the toxic effect of $O_2^{\bullet -}$ and H_2O_2 *in vivo* is due to formation of hydroxyl radical ($^{\bullet}OH$) in a series of reactions catalyzed by transition metal ions. The Haber-Weiss reaction is an iron-catalyzed reaction in which ferric ion (Fe^{3+}) is reduced to

ferrous ion (Fe^{2+}), followed by the Fenton reaction in which Fe^{2+} catalyses the transformation of H_2O_2 into hydroxyl radical $^{\bullet}OH$.

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad \text{Haber-Weiss reaction}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH^{-} + {}^{\bullet}OH \qquad \text{Fenton reaction}$$

An alternative pathway for ($^{\bullet}OH$) production *in vivo* may involve the MPO and EPO. In the presence halides at physiological concentrations, MPO produces hypochlorous acid (HOCl), EPO produces hypobromous acid (HOBr) which can result in the production of $^{\bullet}OH$ in the presence $O_2^{\bullet -}$ (Comhair and Erzurum, 2002).

Hydroxyl radicals can react with different molecules including proteins, DNA and lipids. When the cellular and anatomic sites of production of O_2^- and NO are the same they react together to produce peroxynitrite $ONOO^-$ (Fig. 1.11). Peroxynitrite is a strong oxidant capable of reacting by multiple oxidative mechanisms (van der Vliet and Cross, 2000).

$$O_2^{\bullet -} + {}^{\bullet}NO \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \longrightarrow ONOOH$$

Peroxynitrous acid ONOOH spontaneously decomposes to nitrate (NO_3^-) via the intermediate formation of a nitrogen dioxide (NO_2) and hydroxyl radical. *In vivo*, the reactivity of $ONOO^-$ is quite wide. Its half-life is approximately 1 second in

phosphate buffer at 37°C and pH 7.4, which allows it to diffuse for up to several cell diameters from its site of formation (Zhu *et al.*, 1992).

As stated earlier, peroxynitrite is emerging as a major product among RNS and ROS, not only toward microorganisms but also towards mammalian cells. $ONOO^-$ is also a nitrating agent, since it can react with tyrosine residues in proteins yielding 3-nitrotyrosine, which has been detected in several lung diseases, including acute lung injury, adult respiratory distress syndrome (ARDS), asthma, idiopathic pulmonary fibrosis and CF. This nitration pathway would inhibit tyrosine phosphorylation (Grisham *et al.*, 1999). The electron transfer onto tyrosine residues deforms protein structure and consequently alters functions (van der Vliet and Cross, 2000). In addition, the structure of nitrotyrosine resembles dinitrophenol, which is a strongly antigenic compound used for making haptens. Endogenous nitration may therefore initiate autoimmune processes (van der Vliet *et al.*, 1999).

The deleterious actions of $ONOO^-$ can also be mediated by interactions with several other biological targets, including haem proteins such as cyclooxygenase and lipoxygenase, or nucleophilic targets in protein or DNA. Modification of DNA bases by NO-derived oxidants (purine deamination, guanine nitration) has been implicated in NO-associated mutagenesis (van der Vliet and Cross, 2000). However, $ONOO^-$ can react rapidly with low molecular weight thiols, such as glutathione (GSH) or cysteine forming S-nitrosothiols which have been proposed as a mechanism either of enzyme regulation or NO transport, and may provide a unique signaling mechanism induced by nitrosative stress (van der Vliet *et al.*, 1999; van der Vliet and Cross, 2000). Another newly suggested biological target of the oxidative damage of $ONOO^-$

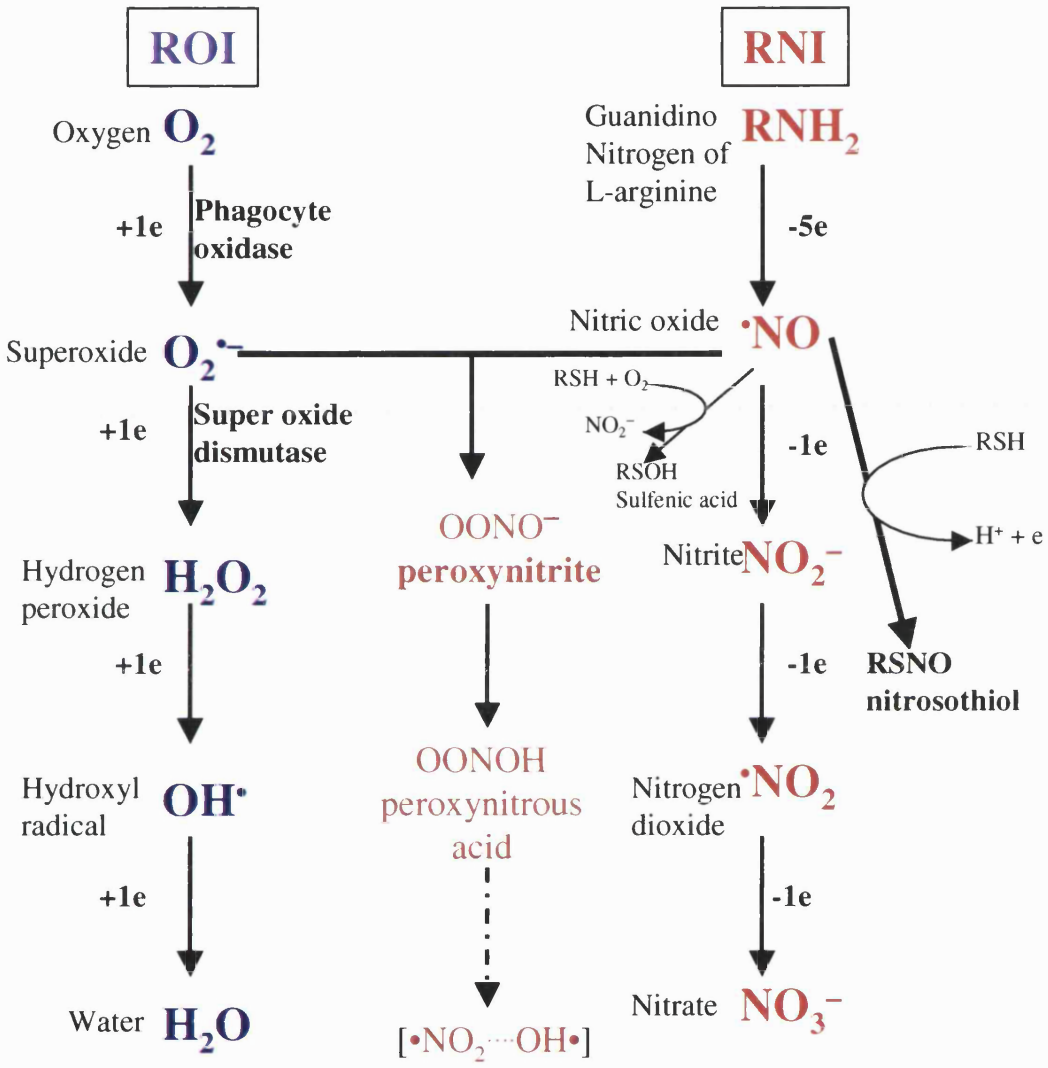


Fig. 1.11. Reactive oxygen species (ROS) and reactive nitrogen inter-species (RNS) production and pathways in mammalian cells.

is methionine (Met). Oxidation of Met residues may alter the biological activity of specific proteins such as ribosomal protein L12, α-1-proteinase inhibitor, calmodulin, and the voltage-gated K^+ channel (St.John *et al.*, 2001).

1.6.2 Cellular antioxidant defence

To minimise oxidant damage to biological molecules, the human lung has anti oxidant activity products that can be acted upon by other antioxidants. Normally, ROS and RNS are removed rapidly before they cause cell dysfunction and cell death. There are a number of primary antioxidant enzymes in the lung including catalase, superoxide dismutase (SOD) and a glutathione system (GSH). SOD is an ubiquitous enzyme with an essential function in protecting aerobic cells against oxidative stress. It catalyses $O_2^{\bullet -}$ to H_2O_2. Catalase is most effective in the presence of high concentrations of small molecules such as H_2O_2 and methyl or ethylperoxide. In the presence of low concentrations of either H_2O_2 or other peroxides, the glutathione system plays a critical role (Comhair and Erzurum, 2002). In addition, nonenzymatic antioxidants called 'scavengers' have a suicide role by reacting directly with oxidizing agents. These include vitaminE (α-tocopherol), β-carotene, uric acid, glucose, bilirubin, taurine, albumine, and cytseine and cysteamine (Comhair and Erzurum, 2002).

In vitro studies suggest that mutation in CFTR may impair lung antioxidant defences making the CF lung more susceptible to oxidative stress (Velsor *et al.*, 2001).

1.7 Bacterial infection of the respiratory tract of CF

Chronic microbial colonization of the major airways is the major source of morbidity and mortality in CF patients. As described earlier, respiratory infection is not the result of altered pulmonary development since lungs of neonates with CF are structurally normal as well as sterile. Rather, inactivation of a first line of defence related directly or indirectly to mutations in CFTR, allows colonization of the respiratory tract with characteristic bacterial species. Initially, *Staphylococcus aureus* and non-typable *Haemophilus influenzae* are frequently isolated from young patients with CF. However, there are no compelling data to indicate that either *S. aureus* or *H. influenzae* contribute to lung function decline in CF except on rare occasions when they cause pneumonia or emphysema (Pier, 2000). These infections typically make the respiratory tract susceptible to the opportunistic pathogen *Pseudomonas aeruginosa*, which is the major problem for most CF patients (Tummler and Kiewitz, 1999). After prolonged infection with *P. aeruginosa*, which usually become mucoid, CF patients become superinfected with organisms such as *Burkholderia cepacia*, *Aspergillus fumigatus* and atypical *Mycobacterium* (Pier, 2000). *Burkholderia cepacia* has been recognized as a major pathogen in patients with CF since the late 1970s (Henrey *et al.*, 2001). The inherent resistance of *B. cepacia* to multiple antibiotics makes eradication of this pathogen from the lungs of infected individuals especially difficult (Tummler and Kiewitz, 1999). High dose and long term antimicrobial therapy have greatly improved the prognosis, however, *B. cepacia* remains a problem.

Recent work has demonstrated strong evidence that *P. aeruginosa* cells exist as a biofilm in CF lung (Singh *et al.*, 2000). Biofilms are sessile microbial communities. They are initiated by surface attachment of individual planktonic bacteria, followed by cell-cell interactions that facilitate development into a matrix of hydrated polymers of an elaborate three-dimensional structure (Costerton *et al.*, 1995; Watnick and Kolter, 2000). The biofilm mode of growth allows bacterial cells to withstand host immune responses, and exhibit increased resistance to antibiotics and biocides when compared with the same cells growing in liquid culture (Xu *et al.*, 2000). *Pseudomonas aeruginosa* utilizes signal molecules called N-acylhomoserine lactones (AHLs) to monitor its population size in a process called quorum sensing (QS) (Van Delden and Iglewski, 1998; Williams *et al.*, 2000; De Kievit and Iglewski, 2000). In addition AHLs are used by *P. aeruginosa* for cell-cell communication (Davies *et al.*, 1998) and recently it was demonstrated that mixed biofilms of *P. aeruginosa* and *B. cepacia* in the lung of CF employ AHL-dependent QS systems to link biofilm formation with the expression of pathogenic traits (McKenney *et al.*, 1995; Geisenberger *et al.*, 2000; Riedel *et al.*, 2001). Communication by AHLs may be of significant importance for the virulence of this association (Davies *et al.*, 1998) and may also promote the chronic inflammation in the CF lung (Favre-Bonte *et al.*, 2002).

1.7.1 *Burkholderia cepacia* complex

Burkholderia cepacia complex (Bcc) describes a diverse group of bacteria whose natural habitats include soil, water, and the rhizosphere of plants. It is an aerobic Gram-negative motile bacillus that formerly belonged to the genus *Pseudomonas*. *B.*

cepacia complex possess an uncommon genome topology of three replicons, which total approximately 7.6 Mb of DNA with a G+C content of approximately 68-70% (Lessie *et al.*, 1996). This highly plastic genome provides *Burkholderia* with high adaptive capacity to survive in extreme environments (Zanetti *et al.*, 2000).

Burkholderia cepacia complex is divided into nine genomic species or genomovars (Govan, 2002), which are phenotypically similar but genetically distinct groups of strains. They have been classified according to DNA-DNA hybridization, whole cell protein pattern similarity and phenotypic markers. Knowing that all strains of the Bcc were clinical isolates from CF patients, however, strains of genomovar II (*B. multivorans*) and III account for over 90% of clinical isolates (Keig *et al.*, 2001; Govan, 2002) and can be further divided into two groups on the basis of *recA* sequence (Parson *et al.*, 2001). It has been recently demonstrated that clinical isolates from Canada are from genomovar III, the most highly transmissible strains with a higher mortality rate than other members of the complex, while genomovar II appears to be nontransmissible (except between siblings) and associated with relatively benign clinical outcomes (Speert *et al.*, 2002).

Interest in these highly versatile microbes stems primarily from their importance as a phytopathogen causing soft rot in onion bulbs, as biocontrol agents in the bioremediation of toxic agents (Melnikov *et al.*, 2000) and as efficient biopesticides (Govan and Vandamme, 1998; Govan, 2000). Whilst *B. cepacia* is an environmental pathogen, there is clear evidence of person-to-person spread of various *B. cepacia* epidemic strains in CF populations both within and outside hospitals. Molecular typing of isolates from patients and contacts demonstrated that transmission is strain-

specific (Cairns *et al.*, 2001). It has been found that clinical isolates can survive for long periods in respiratory droplets on environmental surfaces typically found in CF clinics (Drabick *et al.*, 1996). In addition it was proposed that *Acanthamoeba* and other free-living amoeba can serve as a reservoir for *B. cepacia* and may serve as a vehicle for transmission of *B. cepacia* from patient to patient (Marolda *et al.*, 1999). According to data from North America and Europe, the prevalence of *B. cepacia* in CF clinics is approximately 3-8%, with occasional epidemics increasing the prevalence to around 30% (Fitzgerald *et al.*, 2001). In United Kingdom CF centres, *B. cepacia* has been isolated from up to 40% of CF patients (Hughes *et al.*, 1997).

1.7.1.1 Pathogenesis of *B. cepacia* in CF

For the past two decades *B. cepacia* strains have emerged as human opportunistic pathogens. The most frequently reported site of infection in human is the lower respiratory tract of patients with CF (Govan and Deretic, 1996) or chronic granulomatous disease (CGD), which is a rare genetic disorder resulting in a defect in oxidative killing mechanism of phagocytic cells (Speert *et al.*, 1994). In CF there are two main cell types encountered by *B.cepacia* infection, they are respiratory epithelial cells and pulmonary macrophages where organisms can persist and survive (Burns *et al.*, 1996; Tipper *et al.*, 1998; Saini *et al.*, 1999; Martin and Mohr, 2000). In addition *B. cepacia* organisms have been harvested from tracheal epithelial cells at the time of autopsy from a CF patients (Burns *et al.*, 1992).

Infection with *B. cepacia* in CF is usually acquired late in the course of the disease and can lead not only to a decline in respiratory function, due to onset of necrotizing

pneumonia, but also to an acute systemic infection, such as bacteremia or septicemia (Tablan *et al.*, 1985). The rapid clinical decline due to *B. cepacia* colonization is known as *cepacia* syndrome, which leads to mortality in approximately 20 to 35% of chronically colonized individuals (Isles *et al.*, 1984). Furthermore, infection with *B. cepacia* reduces the life expectancy of CF patients by 50%, from 30 to 15 years (Hutchison and Govan, 1999).

Although fatal pulmonary infections in CF patients are associated with *B. cepacia*, the pathogenic mechanisms and virulence determinants are only beginning to be elucidated. Over 80% of CF patients are typically colonized with *P. aeruginosa*. Indeed, it has been suggested that *P. aeruginosa* produces an extracellular factor, which facilitates the attachment of *B. cepacia* (Riedel *et al.*, 2001). *B. cepacia* appears to establish colonization by adhering first to respiratory tract epithelial cells (Chiu *et al.*, 2001). Attachment occurs via pili (including the *cbl* pilus) that bind specifically to cytokeratin 13 of respiratory squamous epithelial cells (Sajjan *et al.*, 1995), although attachment to non-squamous epithelial cells has been reported (Burns *et al.*, 1996). *In vitro* studies recently indicated that flagellum-mediated motility of *B. cepacia*, may facilitate its penetration of host epithelial barriers, contributing to establishment of infection and systemic spread of the organism (Tomich *et al.*, 2002).

Once within the host, *B. cepacia* could produce a number of potential virulence factors, including proteases (Kooi *et al.*, 1994), lipases (Jørgensen *et al.*, 1991), hemolysins (Nakazawa *et al.*, 1987), siderophores (Darling *et al.*, 1998), catalase, superoxide dismutase (SOD) (Lefebre and Volvano, 2001), melanin like pigment (Zughaier *et al.*, 1999a) and lipopolysaccharide (Zughaier *et al.*, 1999b; Shimomura

et al., 2001) which have been suggested to cause pathological changes in the CF lung. However, little is known about the pathophysiology of *B. cepacia* in CF. Recently a murine pulmonary model of sustained infection was developed to understand better the pathogenicity among genomovars of *B. cepacia* as well as to demonstrate differences in virulence among genomovars (Chu *et al.*, 2002). Colonization of CF patients with *B. cepacia* can result in three clinical outcomes: asymptomatic carriage, slow and continuous decline in lung function, or for approximately 20% of the patients, severe lung dysfunction and fatal pneumonia, the so- called 'cepacia syndrome' (Isles *et al.*, 1984). Epidemiological studies have demonstrated that patients colonized with the identical strain of *B. cepacia* may respond differently, suggesting that cepacia syndrome may not be solely dependent on the type of strain colonization but also to underlying host factors to colonization (Palfreyman *et al.*, 1997). Once colonization with this pathogen is established it is rarely, if ever, be eradicated. A potential explanation is its ability to invade and survive within the respiratory epithelial cells (Burns *et al.*, 1996) and phagocytes (Saini *et al.*, 1999). Intracellular growth in macrophages is an important way in which *B. cepacia* can avoid antibiotic exposure and evade the immune response (Tummler and Kiewitz, 1999). Survival within professional phagocytes suggests that *B. cepacia* can escape the bactericidal mechanisms employed by these cells. *Burkholderia cepacia* demonstrate resistance to non-oxidative killing pathways mediated by cataionic peptides of neutrophils, such as β-defensin, as they are able to survive in the presence of neutrophils from CGD (Speert *et al.*, 1994). In addition *B. cepacia* is resistant to β-defensin-like activity of airway epithelial cells (Baird *et al.*, 1999). Therefore, any

killing of *B. cepacia* by phagocytes must be mediated by ROS. However, Saini and colleagues (1999) demonstrated survival of *B. cepacia* in a macrophages cell line, in the presence of cell activation and oxidative burst, suggesting that bacterial resistance to oxidative damage may explain in part, their persistence and ability to elicit a continual inflammatory response (Saini *et al.*, 1999). Martin and Mohr, (2000) established a macrophage model of invasion and intracellular survival for *B. cepacia,* which was found enclosed in a membrane-bound vacuole (Martin and Mohr, 2000). It has recently been shown that the melanin-like pigment produced by epidemic strains of *B. cepacia* is able to scavenge superoxide anions, which may allow it to survive the respiratory burst response of phagocytic cells (Zughaier *et al.*, 1999). Furthermore, a recent study showed that NO acts synergistically with ROS to kill *B. cepacia in vitro* (Smith *et al.*, 1999). Since iNOS activity may be absent from cells in CF patients (Kelley and Drumnn 1998), this may explain the survival of *B. cepacia* in the CF lung. However, intracellular survival does not appear to be a function common to all strains of *B. cepacia* CF (Martin and Mohr, 2000). Differences in the ability to survive intracellularly may have implications for the varied disease progression associated with *B. cepacia* infections in CF (Chu *et al.*, 2002).

Aims and objectives

Increasing susceptibility of CF patients to bacterial infection may be attributed to two main factors. First, underlying host factors that permit initial colonization of the CF lung by pathogenic bacteria, despite a vigorous host immune response. Secondly, the ability of certain opportunistic bacteria to persist and survive intracellularly in human cells. The aims of this study were:

- To investigate the intracellular signaling mechanisms involved in nitric oxide production in human pulmonary epithelial cells by pro-inflammatory cytokines.

- To determine if substrate availability may play a role in controlling the rate of nitric oxide production.

- To determine the susceptibility of strains of *B. cepacia* complex to hydrogen peroxide at two different stages of life cycle (mid-log and early stationary) when grown as planktonic and biofilm forms.

- To determine catalase activity in the some strains *B. cepacia* complex when growing as planktonic and biofilm.

- To isolate, clone and sequence the catalase gene (*katE*) of *B. cepacia* J2315 that belongs to genomovar III.

2. Methods

2.1 Materials

All reagents were supplied by Sigma Chemical Co. (Poole, Dorset) unless otherwise stated. Electrophoresis-grade agarose, EDTA, ethanol, isopropanol, $MgCl_2$ and $MnCl_2$ were supplied by Merck (Poole, Dorset). All media and reagents used for cell culture were supplied by Gibco BRL (Paisley, Scotland).

2.2 Cell Culture

A549 cells, obtained from American Type Culture Collection (ECACC), were routinely cultured in $80cm^2$ tissue culture flasks in Dulbecco's modified Eagles medium (DMEM) supplemented with penicillin (10 units/ml); streptomycin (10 μg/ml), fungizone (0.5 μg/ml) and 10% (v/v) heat inactivated foetal bovine serum (FBS), which will be referred as complete medium. Cultures were maintained at 37°C in an atmosphere of 5% CO_2. Cultures were fed every three days. Confluent monolayers were used to subculture cells where they were washed three times with Dulbecco's phosphate-buffered saline (DPBS) (without Ca^{2+} and Mg^{2+}). Then cells were incubated approximately for 5 min, until cells had detached from the flask, at 37°C with 1 ml Trypsin-EDTA mixture of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA. So as to inhibit the action of Trypsin-EDTA 25 ml complete medium were added to stop the action of Trypsin-EDTA after complete detachment. The cell suspension was centrifuged at 2000 × g for 5 minutes. The cells pellet was resuspended in 5 ml complete

medium and cell counting and viability were checked in a Naubauer haemocytometer after mixing with trypan blue (0.4% w/v in PBS). Cells were seeded at 10^5/ml of DMEM complete medium into 80 cm^2 tissue culture flasks or into 6 well plates for experimental procedures.

For storage, cells were resuspended at 4×10^6 cells/ml of freeze medium (10% (v/v) DMSO, 40% (v/v) FBS, and 50% (v/v) DMEM). The cell suspension was transferred to cryotubes at 1 ml/tube. Cells were then cooled gradually in the vapor phase overnight and then finally were stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells from one cryotube were rapidly defrosted at 37°C in water bath, washed and resuspended in DMEM complete medium and then seeded into 80cm^2 tissue culture flasks in DMEM complete medium.

2.2.1 Experimental protocol

For all experimental procedures A549 cells were grown until confluent. Prior to experiments, monolayers were washed and cultured in FBS-free DMEM for 24 hours. Growth arrested cultures were treated with fresh FBS-free medium and stimulated with the appropriate doses of either drugs, cytokines or vehicle controls for the time described. Supernatants were collected, centrifuged to remove cellular debris and stored at -70°C until assayed for extracellular nitrite (see below).

2.2.2 Fluorometric nitrite assay

Nitric oxide (NO) production by A549 cells was determined by measuring the stable end product nitrite in the cell culture supernatants by fluorometric assay. It was based upon the reaction of nitrite, under acidic conditions, with 2,3-diaminonaphthalene (DAN) (Lancaster Synthesis Ltd.) to result in the formation of the fluorescent product 1-(H)-naphthotrizole. The assay was modified from the method of Miscko *et al.*, (1993) for use on a Photon Technology International (PTI) spectrofluorimeter. As specified by Misko *et al.*, (1993), an excitation wavelength of 365 nm and an emission wavelength of 405 nm were used. A standard curve of sodium nitrite in FBS-free DMEM ranging from 20 nM to 3.0 μM was prepared. The reaction was initiated by mixing 1 ml standard or culture supernatant with 100 μl of freshly prepared DAN reagent (50 μg/ml DAN in 0.62 M HCl) in disposable cuvettes. The mixture was incubated at room temperature in the dark for 10 minutes and the reaction was stopped by the addition of 1 ml of 0.28 N NaOH. The samples were read in spectrofluorimeter and nitrite concentration was calculated by reference to the standard.

2.2.3 Cell lysis and sample preparation

After each specified treatment of confluent monolayers of A549 cells in 6-well plates culture, stimulations were determined at the appropriate times by aspiration of supernatants. The cells were rinsed with ice-cold Dulbecco's phosphate-buffered saline (DPBS) twice. Three hundred microliter of ice-cold lysis buffer were added to each sample containing 50 mM tris (hydroxymethyl)aminomethane (Tris-HCl)(pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol,

1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (Chang *et al.*, 1998). Cells were disrupted using a cell scraper and the resulting lysates transferred to microfuge tubes, which were incubated at 4°C for 15 minutes. Cells were lysed by sonication at a frequency of 20 KHz (Soniprep 150) for 30 seconds (10 second/cycle) in an ice water bath. Finally, lysates were centrifuged at 12000 × g for 10 minutes at 4°C. Supernatants were transferred to fresh 1.5 ml microfuge tubes and kept on ice until assayed.

2.2.4 Arginase assay

Arginase activity in cell lysates was measured from the rate of urea production using the method descried by Corraliza *et al.*, (1994). Fifty micoliters of cell lysate were added into 50 μl Tris-HCl (50 mM; pH 7.5) containing 10 mM $MnCl_2$. A549 arginase was then activated by heating this mixture at 55-60°C for 10 minutes. The hydrolysis of L-arginine by arginase, was carried out by incubating the mixture containing activated arginase with 50 μl of L-arginine (0.5 M; pH 9.7) at 37°C for 1 hour and was stopped by adding 200 μl of acid solution mixture (H_2SO_4:H_3PO_4:H_2O = 1:3:7). For colorimetric determination of urea, α-isonitrosopropiophenone (25 μl, 9% in absolute ethanol) was added, and the mixture was heated at 100°C for 45 minutes. After the mixture was incubated in the dark for 10 minutes at room temperature. The urea concentration was determined spectrophotometrically by the absorbance at 540 nm measured with a Dynatech MR5000 microplate reader, using 200 μl aliquots in non-sterile microtiter plate. A standard curve was prepared with increasing amounts of urea between 0.1 and 0.9 μmol.

2.3 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables 2.1. *B. cepacia* and *E. coli* cultures were routinely grown in Luria-Bertani (LB) medium (Bacto-tryptone 10 g/l, yeast extract 5g/l, NaCl 5 g/l, pH 7.5) at 37°C with aeration (200 rpm), or on agar plates containing LB medium supplemented with 1.5% Bacto-agar and incubated at 37°C for the time required. The medium was supplemented with ampicillin 100 μg/ml as required.

	Description	**Source/reference**
Burkholderia cepacia J2315	Genomovar III	Smith *et al.*, 1999
Burkholderia cepacia J2552	Genomovar I	Smith *et al.*, 1999
Burkholderia cepacia JL27	Genomovar III	Smith *et al.*, 1999
Burkholderia cepacia JL32	Genomovar III	Smith *et al.*, 1999
Escherichia coli JM109	Amp^r	Promega

Table 2.1. List of bacterial stains.

2.3.1 Maintenance of strains

Strains were stored at -70°C in LB with 20% (v/v) glycerol. Bacteria were revived by streaking aliquots on appropriate media and incubating at 37°C. Short-term working stocks were maintained at 4°C on solid media. *B. cepacia* strains were kept at room temperature.

2.3.2 Measurement of optical density

Growth of bacterial cultures was routinely monitored by measuring optical density at 470 nm in a Spectronic 601 spectrophotometer (Milton Roy).

2.3.3 Inoculation and growth of planktonic cultures

Cultures were grown at 37°C with aeration (200 rpm) in baffled, 250 ml conical flasks. Overnight starter cultures were grown in 25 ml volumes and all other cultures were grown in 25 ml or 50 ml volumes. A standard inoculation procedure was followed; overnight cultures were diluted in a ratio of 1:100 into fresh LB broth.

2.3.4 Inoculation and growth of biofilm cultures

Biofilms were grown on a filter support placed on LB agar plates. Sartorius hydrophobic edged membranes (pore size 0.2 μm, 45 mm diameter) (Fisher Scientific) were placed in a filter holder (Nalgene) and washed with 5 ml sterile M9 minimal salts solution (Buhler *et al.*, 1998). Membranes were then placed on the surface of LB agar and prewarmed at 37°C for 1 hour. Cells from stationary phase culture were diluted to 2×10^7 ml^{-1} in sterile M9 minimal salt solution. An aliquot (50 μl) of the bacterial suspension was dropped onto the centre of the membrane and the agar plate/membrane was incubated at 37°C. Filter grown cells were harvested by scraping the membrane with a sterile glass spreader, resuspended in 5 ml M9 minimal salts solution and vortexing to dispense aggregates.

2.3.5 Bacterial killing assay

Bacterial cultures were grown in LB broth or on filter membranes at 37°C until stationary required phase. For planktonic cultures they were harvested by membrane filtration, washed and suspended in M9 minimal salt solution to 1×10^9 cfu/ml, while for membrane cultures a bacterial suspension of 1×10^9 cfu/ml was prepared directly. Bacterial cells were added to 5 mM H_2O_2 to a final density of 1×10^7 cfu/ml. Samples were removed every 15-minutes period for 60 minutes, and put into an inactivation medium comprising catalase 10 000U/ml in M9 salts solution. A minimum of a 1 in 10 dilution into inactivation medium was performed. After appropriate serial dilution in this inactivation medium 10 µl samples were inoculated in triplicate on LB agar plates using the Miles and Misera, (1938) method. Colony-forming units (cfu) were counted after 48 hours incubation at 37°C, and the $\log_{10}$ reduction in viability calculated against a non-treatment control.

2.3.6 Detection of bacterial catalase activity

2.3.6.1 Preparation of crude cell extract

Cell extracts were prepared from 30 ml planktonic cultures, which were centrifuged at 7000 × *g* for 10 minutes at 4°C. Bacterial pellets were washed twice in ice-cold 0.05 M potassium phosphate buffer (pH 7.0) and finally resuspended in 1.5 ml 0.05 M potassium phosphate buffer (pH 7.0) supplemented with 0.1% dithiothreitol stored on ice. Cells were lysed by sonication (Soniprep 150) for a total of 3 minutes (60 second/cycle) in an ice water bath.

Lysates were centrifuged at 12 000 × *g* for 5 minutes at 4°C to sediment cellular debris and unbroken cells. Supernatants were stored at -20°C in 50% (v/v) glycerol. Under these conditions catalase activity could be preserved for several months.

For the preparation of a crude cell extracts from bacterial biofilms the same procedure was used. In order to obtain sufficient cell mass, extracts were prepared after harvesting 6 inoculated membranes/strain at specific growth points in 30 ml ice-cold 0.05 M potassium phosphate buffer (pH 7.0).

2.3.6.2 Enzymatic activity assay

The catalase specific activities of cell lysates were determined using the spectrophotometric protocol mentioned by Katsuwon and Anderson, (1992). Catalase activity was monitored by following the decomposition of 5 mM H_2O_2 in 0.05 M potassium phosphate buffer at 240 nm at 25°C. An appropriate amount of cell–free extract was added to 650 µl of 5 mM H_2O_2 in 0.05 M potassium phosphate buffer in a quartz cuvette, mixed thoroughly, and H_2O_2 decomposition monitored for 1 minute. Specific activity was calculated from the following equation:

$$\text{Specific activity} = \frac{\Delta OD_{240}/\text{min} \times \text{total reaction volume}}{\varepsilon_{240} \times \text{mg protein}}$$

The ε_{240} is the extinction coefficient = 43.6 M^{-1}. cm^{-1} for H_2O_2.

Where 1 unit of catalase will decompose 1 µmol of H_2O_2 per milligram of protein per minutes at 25°C.

2.3.6.3 Protein assay

The estimation of total protein per lysate was based on the Bradford dye-binding procedure (Bradford, 1976). A 1 mg/ml solution of bovine serum albumin (BSA) was prepared in 0.05 M potassium phosphate buffer (pH 7.0) to produce known concentrations of BSA in 1 ml of Bio-Rad protein assay (Bio-Rad). Equal volumes of each sample (20 µl) were added to 1 ml of Bio-Rad protein assay and 200 µl of sample or standard were transferred in duplicate to the wells of 96-well microtiter plate. The plate was read at 595 nm on a Dynatech MR5000 plate reader. The protein concentrations were calculated by linear regression from the standard curve.

2.3.6.4 Native polyacrylamide gel electrophoresis (PAGE) to visualize catalase isozyme patterns

The native gel electrophoresis method described by Hassett *et al.*, (1999b) was used with Bio-Rad Mini Protean II system. Native 4% (w/v) stacking and 7% (w/v) resolving polyacrylamide gels were used. The wells were loaded with 10-20 µg of cell-free extract per lane after being mixed with high-density sucrose tracking-dye (5 g sucrose, 10 mg bromophenol blue, 1.5 ml running buffer and Milli-Q up to 5 ml). A constant current of 20 mA was applied at 4°C in native PAGE running buffer (25 mM Tris, 192 mM glycine) until the tracking dye reached the bottom of the gel.

After electrophoresis, the gels were stained according to the method previously described by Clare *et al.* (1984). This method involves soaking the gel first in horseradish peroxidase 50 µg/ml in potassium phosphate buffer (pH7.0) for 45 minutes. Hydrogen peroxide was then added to a concentration of 5 mM and soaking continued for 10 minutes. The gels were then rapidly rinsed twice with Milli-Q water and finally placed into 0.5 mg/ml of diaminobenzidine in potassium phosphate buffer (pH 7.0), until staining was completed.

Reagents	**7% Resolving Gel**	**4% Stacking Gel**
40% (w/v) acrylamide:bisacrylamide 37.5 : 1	3.5 ml	1.33 ml
1 M Tris-HCl , pH 8.8	5.6 ml	-
1 M Tris-HCl , pH 6.8	-	1.25 ml
Milli-Q Water	6.1 ml	6.51
10% APS	50 µl	50 µl
TEMED	20 µl	20 µl

Table 2.2. Solutions for 7% native-page gel electrophoresis.

2.4 Molecular manipulation

2.4.1 Multiple sequence alignment construction

Sequence alignment was undertaken to analyse the degree of conservation of the catalase KatE peptide sequence of *E. coli*, with sequences of other catalase KatE peptides from Gram-negative bacteria in the Genbank database. Sequence retrieval was performed using database searching at Genbank. A multiple sequence alignment was compared and analyzed using the program Clustalx, which is available on the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk/). After highlighting regions of high homology between KatE catalase peptide sequences of *E. coli* and *P. aeruginosa*, degenerate primers were designed using *P. aeruginosa* codon usage data (West and Iglewski, 1988) and used for PCR amplification of *B. cepacia* chromosomal DNA.

2.4.2 Small scale chromosomal extraction

Small-scale chromosome extraction was carried out using the Genomic kit from Helena Biosciences (Sunderland, UK), in accordance with the manufacturer's instructions.

2.4.3 Spectrophotometric determination of nucleic acid concentration

Quantitation of DNA was performed using a GeneQuant II spectrophotometer (Pharmacia Biotech, St. Albans, Herts). Then nucleic acid concentration was determined from absorbance at 260 nm; the ratio of absorbance at 260 nm and 280 nm was used as an indicator of nucleic acid purity.

2.4.4 Polymerase chain reaction (PCR)

Reaction mixtures of a total volume of 100 µl were prepared with 200 µM each of deoxynucleotides, 2 mM magnesium chloride, 10 µM primers and 10 µl 10 × reaction buffer (all supplied by Gibco BRL, Paisley, Scotland). Template DNA (50 µg of genomic DNA, or 10 ng plasmid DNA) was added and 1 unit of Platinum *Taq* DNA polymerase (Gibco BRL) used in each reaction. Each reaction was overlaid with 59 µl of mineral oil to prevent evaporation. PCR was carried out in a Crocodile II thermocycler (Appligene, Watford, UK) with an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minutes) and extension (72°C, 1 minute per 1 kb). The reaction was terminated with an extended elongation step (72°C for 5 minutes). PCR products were analyzed by horizontal agarose gel electrophoresis.

2.4.5 Horizontal agarose gel electrophoresis

Horizontal agarose gel electrophoresis was used to resolve DNA fragments between approximately 100 bp and 10,000 bp. Electrophoresis was performed in a Bio-Rad DNA subcells (Bio-Rad Richmond, Ca.). The running buffer comprised 1× TBE (Tris/borate/EDTA). Electrophoresis-grade agarose (0.7% to 1.5%) was dissolved in an appropriate volume of 1× TBE buffer at high temperature in a microwave oven. The solution was allowed to cool to approximately 60°C before being poured into a casting tray and left to set. The gel tray was mounted in an electrophoresis tank and submersed in 1× TBE.

DNA samples were mixed with 6× loading buffer (10 mM Tris, 25% glycerol, 0.25% bromophenol blue, 0.5% SDS, 0.05 M EDTA) in a 5:1 a ratio and loaded onto the gel. Appropriate DNA standards were also loaded (Helena BioSciences). Generally, a 1 kb ladder was used for DNA samples >1 kb, and a 100bp ladder for samples < 1 kb. Electrophoresis was carried out at 80 V for 90 minutes or until full separation of bands, after which the gel was soaked in an ethidium bromide (0.5 μg/ml) solution 30 minutes. The stained gel was visualized on a long wave 360 nm UV transilluminator and photographs taken using a Polaroid MP4 camera and Polaroid 637 film.

2.4.6 Extraction of DNA from agarose gels

DNA was recovered from agarose gels using the QIAquick Gel Extraction kit (Qiagen, UK), in accordance with the manufacturer's instructions.

2.4.7 Ligation of plasmid DNA

Ligation of vector and foreign DNA was carried out as described by Sambrook *et al.*, 1989. Reactions were set up by mixing 50 ng of vector DNA with an equimolar amount of insert DNA. The following equation was used to determine the required amount of insert DNA:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ration} = \text{ng of insert}$$

The ligation reaction mixture was made up to a final volume of 10 µl by the addition of 1 µl DNA ligase buffer (10×), 1 µl of 1 U/µl T4 DNA ligase (Helena BioSciences) and Milli-Q water. The reaction was incubated at 16°C for 1 to 16 hours. Usually, 1 to 2 µl of each ligation reaction used to transform *E. coli* cells.

The pGMT-T Vector System (Promega) was used since it is a convenient system for cloning PCR products. This plasmid is provided in a linearized form with a single thymidine overhang at both its 3'-ends. The presence of these overhangs greatly improves the efficiency of ligation of PCR products, which possess complementary 3'-adenine overhangs. Ligation reactions were carried out as instructed by the manufacturer.

2.4.8 Preparation of competent *E. coli* JM109 cells

A single fresh colony of *E. coli* JM109 was inoculated into 5 ml LB broth and incubated overnight at 37°C with aeration (200rpm). This primary culture was diluted 1:100 and grown until the OD_{470} reached 0.48. The suspension was chilled on ice for 5 minutes, and then centrifuged at 4,000× *g* for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in $2/5^{th}$ volume of transforming buffer I (30 mM KCl, 100 mM RbCl, 10 mM $CaCl_2$, 50 Mm $MnCL_2$, 15% v/v glycerol, pH 5.8) and chilled on ice for 5 minutes. The cells were centrifuged at 4,000× *g* for 5 minutes at 4°C and supernatants discarded. The cells were then resuspended in $1/25^{th}$ volume of transforming buffer II (10 mM MOPS, 75 mM CaCl2, 10 mM RbCl, 15% v/v glycerol, pH 6.5) and left on ice for 15 minutes. Finally, the cell suspension was divided into 200 µl aliquots and snap frozen on dry ice/ethanol and stored at -70°C until required.

2.4.9 Transformation of competent *E. coli* JM109 by heat shock

Competent cells were thawed at room temperature and then chilled on ice for 10 minutes. The DNA was added and the suspension was mixed gently and left on ice for 30-45 minutes. Heat shock was carried out at 42°C for 2 minutes and then immediately cells were cooled on ice for 2 minutes. Four volumes of LB broth were added and the suspension was incubated at 37°C for 1 hour. The cells were pelleted and suspended in 100 µl LB broth. All of the mixture was plated out onto LB agar plates containing appropriate antibiotic.

2.4.10 Blue/white selection

For growing and screening of transformed bacteria containing the correct insert, selective plates were spread with 4 µl of 0.2 mM isopropyl-β-D-thiolgalactoside (IPTG) and 40 µl of X-Gal (5-bromo-4-chloro-3-indoyl β-D galactopyranoside) (20 mg/ml in dimethylformamide). The plates were dried at 37°C for 4 hours. Bacterial colonies containing the recombinant plasmid appeared white, whilst those having the intact *lacZ* gene appeared blue.

2.4.11 Preparation of plasmid DNA

Small-scale plasmid extraction was performed using the Wizard Plus SV Mini Prep kit (Promega, Madison, WI), in accordance with the manufacturer's instructions.

2.4.12 Purification of DNA by ethanol precipitation

Ethanol precipitation was used to clean up and/or concentrate DNA. For the DNA sample 0.5 volumes of ammonium acetate (7.5 M) and 2.5 volumes of 95% ethanol were added to 1 volume of sample. The resulting mixture was centrifuged at 14,000× *g* for 15 minutes, after which the supernatant was discarded. The pellet was then rinsed in 250 µl of 70% ethanol and centrifuged at 14,000× g for 5 minutes. Finally, the ethanol was removed, the pellet air-dried and the DNA suspended in 10-25 µl of TE (Tris/EDTA) buffer pH 8.0 or water.

2.4.13 Restriction plasmid DNA

DNA restrictions were carried out using enzymes purchased from Fermantas. A 20 µl or 50 µl reaction containing the DNA, the enzyme (5-20 U/µg plasmid DNA) and an appropriate buffer, were incubated at 37°C for 1 to 4 hours. The reaction was terminated by addition of 12.5 mM EDTA (pH 8.0) or by heating the solution to 65°C for 20 minutes. Digests were analyzed by agarose electrophoresis.

2.4.14 Sequencing DNA constructs and computer assisted sequence analysis

DNA Sequencing was carried out at the automated DNA sequencing facility in the Department of Biology and Biochemistry at the University of Bath. Sequence analysis was performed using the Wisconsin Genetics Computer Group (GCG) software package on the University of Bath GNOME Unix server. The BLAST search facilities of the National Library

of Medicine, Washington, DC (NCBI) (http://www.ncbi.nlm.nih.gov), were used to search for homologues.

2.5 Methods of statistics

Statistical analysis was used in two contexts in this work. Firstly, data for nitrite production by A549 cells were assessed by 2-way analysis of variance, followed by Dunnett's test (multiple comparisons to control group) or Tukey's test (comparison at all means) as appropriate. *P* value of less than 0.05 was considered significant. Secondly, for bacterial killing experiments significant differences between killing activities in different isolates was assessed by paired or unpaired t-tests on $\log_{10}$ transformed data. Again, *p* value of less than 0.05 was considered significant.

3. Modulation of inducible nitric oxide synthase expression

3.1 Background

The inflamed pulmonary epithelium is an important source of inducible nitric oxide synthase in the lung, which will lead to a tonic expression of NO detected in exhaled air. This suggests that iNOS activity has regulatory roles in pulmonary epithelial cells, which can range from bactericidal activity to the regulation of gene expression. This emphasizes the significance of the detailed understanding of intracellular events regulating the expression of iNOS in human respiratory epithelial cells as a prerequisite to understanding the role(s) of iNOS in lung biology and disease. The vulnerability of CF patients to chronic pulmonary bacterial infections has been attributed, in part, to alterations in NO production. Various reports have demonstrated that the iNOS isoform is constitutively expressed in tracheal and nasal epithelial cells in normal human (Guo *et al.*, 1995). However, Kelley and Drumm, (1998) clearly showed, through immuno-staining, that iNOS is absent in the epithelium of CF airway. Their findings offered a possible explanation for the lack of exhaled NO in CF expired air. Conversely, other investigations did not agree on an explanation for the lack of exhaled NO from CF patients (Robbins *et al.*, 2000; Jones *et al.*, 1998), and attributed this to other phenomena not associated with dysregulation of iNOS. Some studies did not find a decrease in NO from orally exhaled air in CF patients. However, exhaled NO gas may not be a good marker for iNOS activity in the CF airway (Linnane *et al.*, 1998), and there was clearly a lack of increased NO levels that would be expected in an

inflammatory disease (Grasemann *et al.*, 1997; Kelley and Drumm, 1998). *In vitro* studies using human colon epithelial cells revealed that IL-13 downregulates iNOS expression via upregulation of phosphatidylinositol 3-kinase (Wright *et al.*, 1997).

3.2 Results

3.2.1 Cytokine-induced iNOS activity

Using A549 cells as model of human pulmonary epithelial cells, initial investigations sought to confirm the mechanisms involved in nitric oxide production and regulation by T cell-derived cytokines.

The approach undertaken to investigate the regulation of iNOS in human pulmonary epithelium was to investigate firstly that A549 cells produce elevated levels of NO in response to treatment with pro-inflammatory cytokines and secondly, mechanisms modulating iNOS activity.

Growth arrested monolayers of unstimulated A549 produced a small constitutive amount of nitrite, the stable breakdown product of NO (Fig 3.1). To determine whether the individual stimuli would induce iNOS, the pro-inflammatory cytokines IL-1β (0.3 ng/ml), TNF-α (30 ng/ml) and IFN-γ (50 ng/ml) added individually did not induce significant increases in nitrite generation after 24 hours compared with the constitutive nitrite basal level in untreated A549. The combination of IFN-γ/IL-1β was the minimal requirement to induce nitrite production, whilst other pairs of cytokines caused a slight increase in nitrite levels. Stimulation by IFN-γ/ IL-1β produced a moderate increase in nitrite production of 1.75 ± 0.2 μM (n=3). The addition of IFN-γ to the combination of

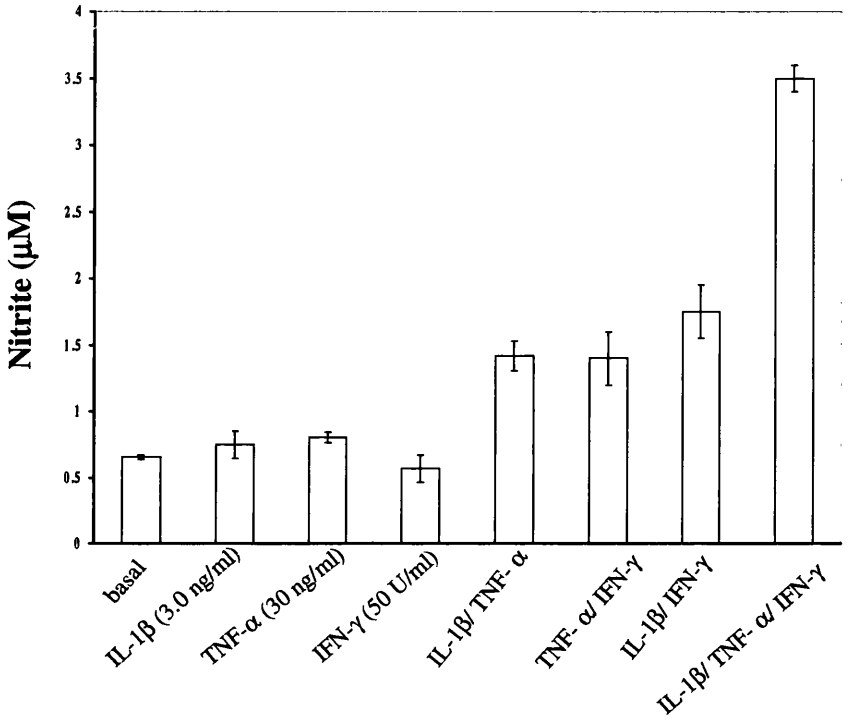


Fig. 3.1. Nitrite production by A549 cells stimulated with cytokines, as indicated for 24 hours. Each point represents the mean ± SEM of at least three experiments.

IL-1β/TNF-α resulted in a significant increase in nitrite. The combination of three cytokines resulted in an approximates of 3-fold enhancement in nitrite levels, i.e. 3.5 ± 0.1 μM (n=3) (Fig.3.1).

Based on dose response studies, there was a concentration dependent increase in nitrite production at different levels by the pair-wise combination of TNF-α/IFN-γ, IL-1β/IFN-γ, after 24 hours of induction (Fig 3.2A and B, respectively). Each of IL-1 β (0.1 - 10 ng/ml) or TNF-α (1 - 100 ng/ml) was used simultaneously with IFN-γ (50 U/ml) and nitrite levels were enhanced as doses of each of IL-1β or TNF-α increased. However, the synergy of the IL-1β /IFN-γ or TNF-α /IFN-γ was mild and nitrite levels at maximal concentrations of IL-1β (10 ng/ml) or TNF-α (100 ng/ml) were 1.75 ± 0.25 and 1.42 ± 0.11 μM, respectively (Fig. 3.2A and B). Accordingly, the sub-maximal concentrations of IL-1β (3 ng/ml) and TNF-α (30 ng/ml) were selected to test for the synergy of IL-1β and TNF-α with IFN-γ (0.5 - 100 U/ml). Interestingly, different concentrations of IFN-γ (0 - 100 U/ml) in the presence of the combination of IL-1β (3 ng/ml) and TNF-α (30 ng/ml) induced a concentration-dependent enhancement of nitrite production (Fig. 3.2C). The sub-maximal concentration of IFN-γ (50 U/ml) was selected and used in subsequent experiments.

Stimulation of A549 cells with IL-1β (3 ng/ml), TNF-α (30 ng/ml) and IFN-γ (50 U/ml) induced a time–dependent generation of nitrite at 4 (0.994 ± 0.025), 8 (1.55 ± 0.24), 12 (1.86 ± 0.29), 24 (2.4 ± 0.44) and 48 (3.14 ± 0.48) hours compared with basal values of 0.74 ± 0.5 μM (Fig. 3.3).

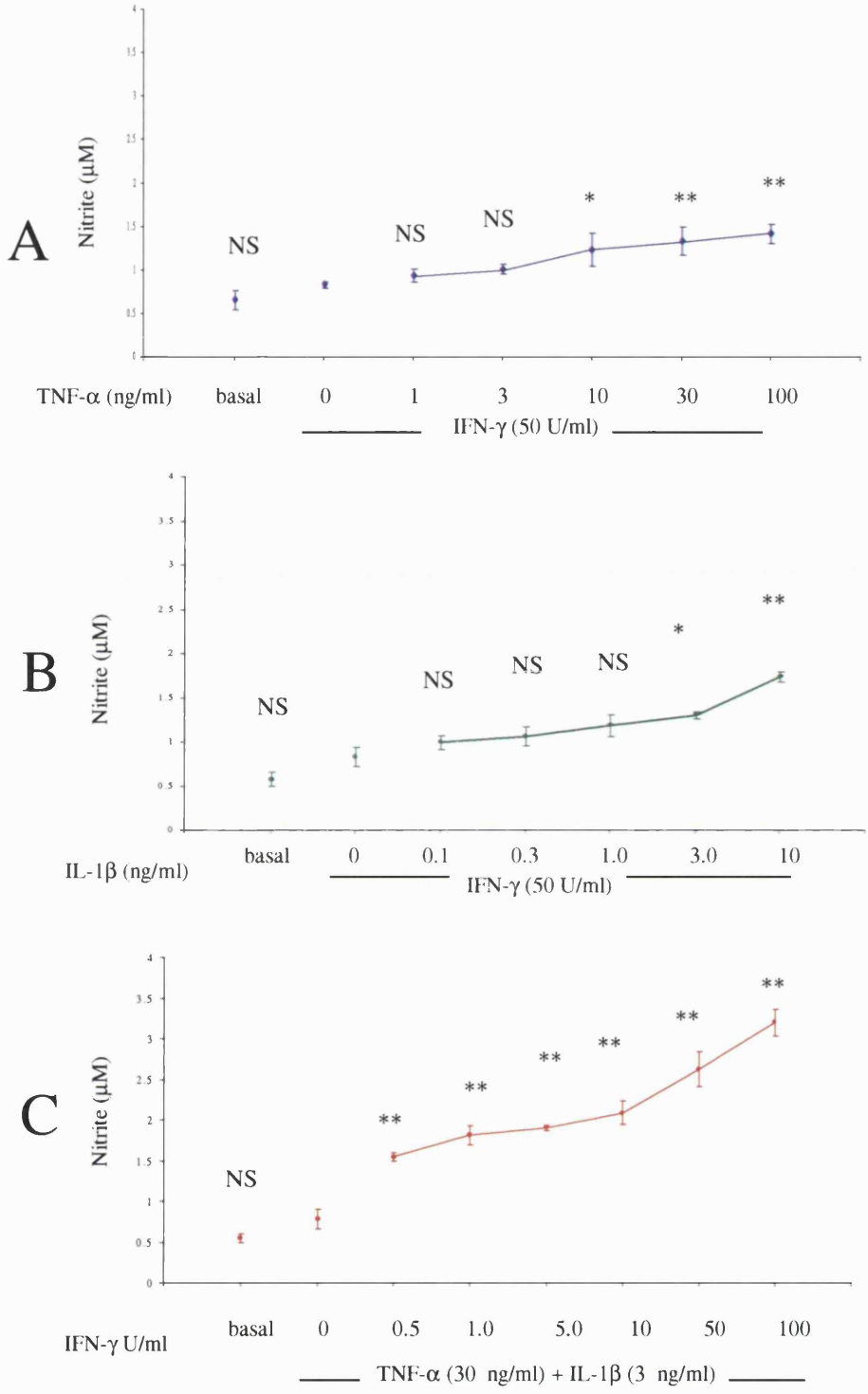


Fig 3.2. Nitrite production by A549 cells after 24 hours treatment with different concentrations of (A) TNF-α (0-100 ng/ml) in the presence of IFN-γ (50 U/ml), (B) IL-1β (0-10 ng/ml) in the presence of IFN-γ (50 U/ml) and (C) IFN-γ (0-100 U/ml) in the presence TNF-α (30 ng/ml) and IL-1β (3.0 ng/ml). Each point is the mean ± SEM of three experiments. Significant nitrite increase compared with 0 value on each figure are indicated by NS (not significant, $p>0.05$), * ($p<0.05$) and **($p<0.01$).

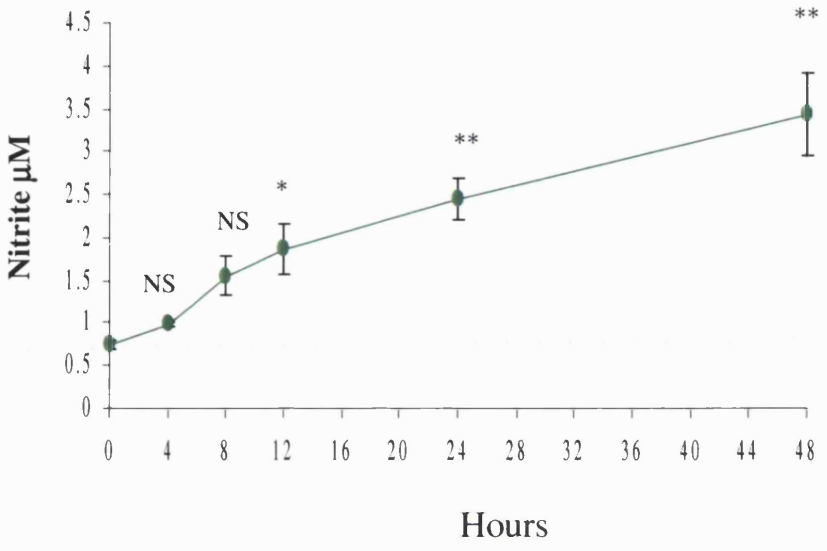


Fig. 3.3. Time course of nitrite production by A549 cells following stimulation with cytokine mixture of TNF-α (30 ng/ml), IL-1β (3.0 ng/ml), and IFN-γ (50 U/ml). Each point represents mean ± SEM of at least three experiments. Significant increase in nitrite production by time incubation compared with 0 hour incubation are indicated by NS ($p>0.05$), * ($p<0.05$) and **($p<0.01$).

3.2.2 Regulation of iNOS activity by NOS substrate inhibitors

Much effort have been given to the design of selective inhibitors of NOS isozymes to be used as biological tools and more importantly as possible pharmacological tools to modulate NO levels. The expression 'selective inhibitor' can be used if the inhibitory effect is against a particular NOS isoform, and inhibition was shown at the isolated enzyme level ideally *in vivo* (Alderton *et al.*, 2001). Thus, 1400W developed by Garvey *et al.* (1997) as a highly selective inhibitor for iNOS, was used to verify that the nitrite measured was the result of NO generated by iNOS activity. Another well-characterised NOS inhibitor used in this study was aminoguanidine. Aminoguanidine has partial selectivity for iNOS versus eNOS, however, its selectivity over nNOS is minimal (Alderton *et al.*, 2001).

Thus, the effects of 1400W and aminoguanidine were measured on cells treated with the cytomix, the combination of cytokines IFN-γ/TNF-α/IL-1β.

Co-stimulation of A549 cells with pro-inflammatory cytokines and iNOS inhibitors resulted in 96 ± 4% (n=3) inhibition of nitrite accumulation by 1400W (10 μM) and by 96 ± 0.7% (n=3) inhibition by aminoguanidine (0.5 mM). However, the inhibitory effect of both 1400W and aminoguanidine (0.5 mM) did not affect the minimal level of nitrite when treated with unstimulated A549. This was indicated with no change to the basal levels of nitrite after 1400W (10 μM) or aminoguanide (0.5 mM) stimulation (Fig. 3.4).

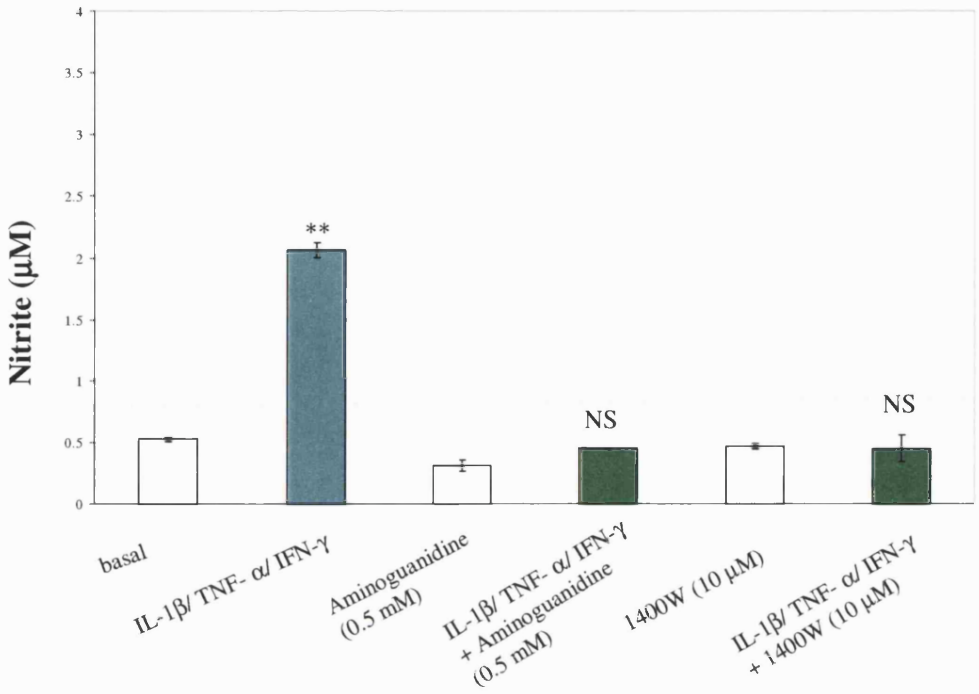


Fig. 3.4. Effect of iNOS inhibitors aminoguanidine (0.5 mM) and 1400W (10 μM) on stimulated A549 with cytokine mixture of TNF-α (30 ng/ml), IL-1 β (3.0 ng/ml), and IFN- γ (50 U/ml) for 24 hours. Each point represents mean ± SEM of at least three experiments. Significant inhibition of nitrite by addition of iNOS inhibitors compared with basal are indicated by NS (p>0.05) and ** (p<0.0001).

3.2.3 Effect of IL-13 on cytokine-stimulated nitrite production

Data indicated that IL-13 could be a potent modulator of immune responses in different types of cells, including suppression of NO synthesis. *In vitro,* IL-13 inhibited iNOS induction in human colon epithelial cells HT-29 (Wright *et al.*, 1997) and in A549 cells (Berkman *et al.*, 1996). Downregulation of pro-inflammatory cytokine induced iNOS in HT-29 cells was via activation of PI 3-kinase by IL-13. Such a scenario of cell signaling, might explain the dysregulation of iNOS in the CF lung. The A549 cell line in this work was used as a model to explore the regulation by IL-13 of pulmonary epithelial iNOS activity. IL-13 had an inhibitory effect on IFN-γ/TNF-α/IL-1β induced nitrite generation. Confluent monolayers of A549 were pretreated for 1 hour with increasing concentrations of IL-13 (0.3 - 30 ng/ml), followed by the combination of cytokines IFN-γ/TNF-α/IL-1β for 24 hours. IL-13 produced a partial but a significant suppression of nitrite levels (Fig. 3.5). In order to test whether IL-13 can inhibit iNOS from stimulated A549 cells via PI 3-kinase signaling, inhibitors were used. The addition of the PI 3-kinase inhibitor wortmannin (10 - 300 nM) to A549 cells 10 minutes before IL-13 treatment partially prevented the inhibitory effect of IL-13 on cytokine-induced nitrite production from iNOS (Fig. 3.6). Similarly, addition of the structurally unrelated PI 3-kinase inhibitor LY294002 (1 – 30 μM) for 15 minutes before IL-13, resulted in partial restoration of nitrite levels from cytokine stimulated A549 cells (Fig. 3.6). Taken together, these data suggest a role for PI 3-kinase in transduction of the IL-13-induced signal, which down-regulates iNOS activity.

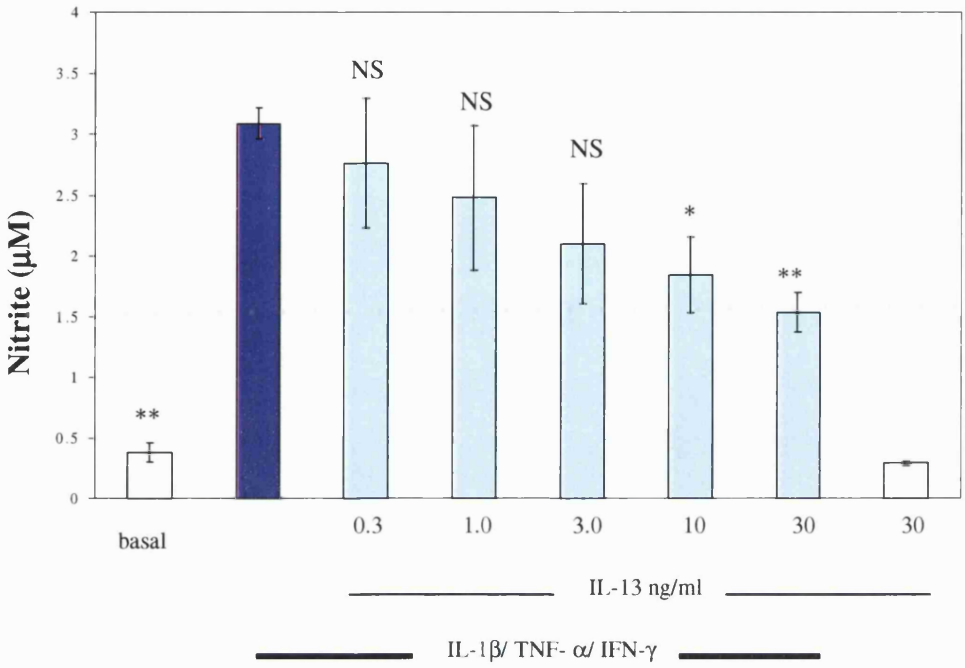


Fig. 3.5. Effect of IL-13 on cytokine stimulated nitrite production in A549 cells. A549 cells were treated with increasing concentrations of IL-13 (0.3-30 ng/ml)for 1 hour prior to the addition of cytokines, as indicated. Each point represents the mean± SEM of three separate experiments. Significant reduction in nitrite levels by different concentrations of IL-13 compared with cytokine stimulated cells are indicated by NS ($p>0.05$), * ($p<0.05$) and **($p<0.01$).

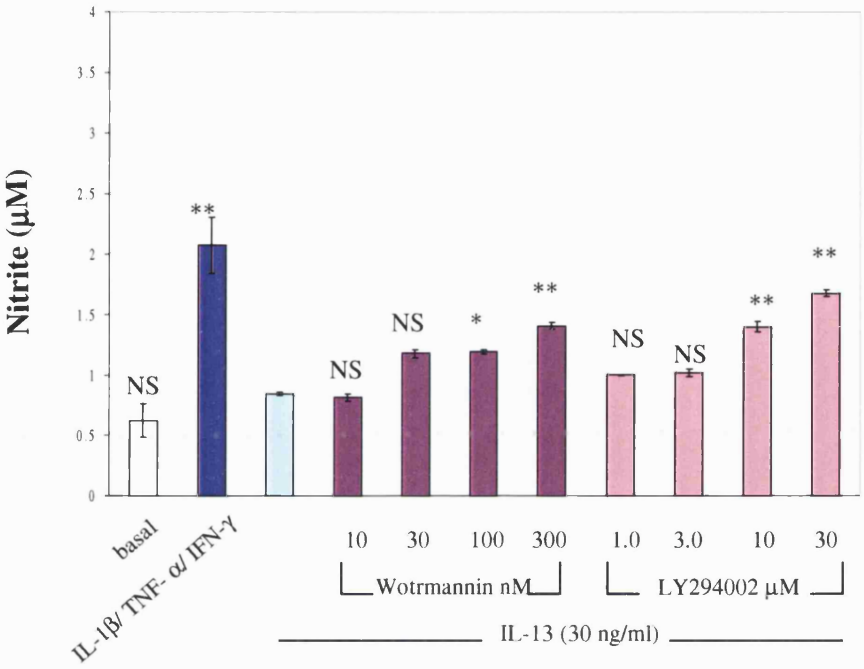


Fig 3.6. Nitrite production by A549 cells after treated with wortmannin for 10 minutes or LY294002 for 15 minutes prior to the addition of IL-13 for 1 hour, followed by cytokines, as indicated, after 24 hours, nitrite in the supernatant was measured using the fluorometric nitrite assay. Data are the mean ± SEM of three experiments. Significant nitrite restored levels after PI 3-kinase inhibiters compared with cytokine stimulated A549 pretreated with IL-13 indicated by NS ($p>0.05$), * ($p<0.05$) and **($p<0.01$).

3.2.4 Regulation of iNOS by substrate limitation

According to Murphy and Newsholme (1998); Chang *et al.* (2000), the lack of NO release by human mononuclear phagocytes and murine macrophages, respectively, is due to an increased breakdown of the NOS substrate L-arginine, by another enzyme, L-arginase. More interestingly, in the recent study by Chang *et al.* (2000) it was demonstrated that activity of IL-13 mediated inhibition of iNOS was via its ability to induce L-arginase. The question then was raised as to whether arginase plays a regulatory role in NO production in A549 cells. Three lines of investigations were taken, firstly a study of the effect of the extracellular L-arginine supply on NO production, secondly assay for arginase activity in A549 and thirdly investigate if IL-13 modulates iNOS expression through arginase induction.

Upon addition of an excess amount of the substrate, L-arginine (2 mM) to the DMEM medium the standard curve for the flourimetric nitrite assay was not altered (Fig. 3.7). However, NO levels increased 39 ± 10% from unstimulated A549, 22 ± 9% from cytokine-stimulated A549 and 32 ± 6% after IL-13 treated cytokine stimulated A549 when grown in DMEM containing an excess of L-arginine (2 mM) for 24 hours. The presence of excess substrate L-arginine, had no significant effect (p = 0.6) on the change of nitrite from cytomix –induced A549 treated with or without IL-13 (Fig. 3.8).

Lysates of unstimulated as well as cytokine-stimulated A549 failed to convert L-arginine to urea after being activated at 55-60°C for 10 min. In parallel there were no detected increases in NO accumulated from cytokine-stimulated and unstimulated A549, after the addition of L-norvaline, which is an arginase inhibitor. To observe dose

dependence of L-norvaline on nitrite accumulation from cytokine-stimulated A549, increased concentrations of L-norvaline (5-20 mM) added resulted in no increase in nitrite accumulation from cytokine stimulated A549 (Fig. 3.9). The addition of L-norvaline (20 mM) did not reverse IL-13 inhibition of nitrite production (Fig. 3.10).

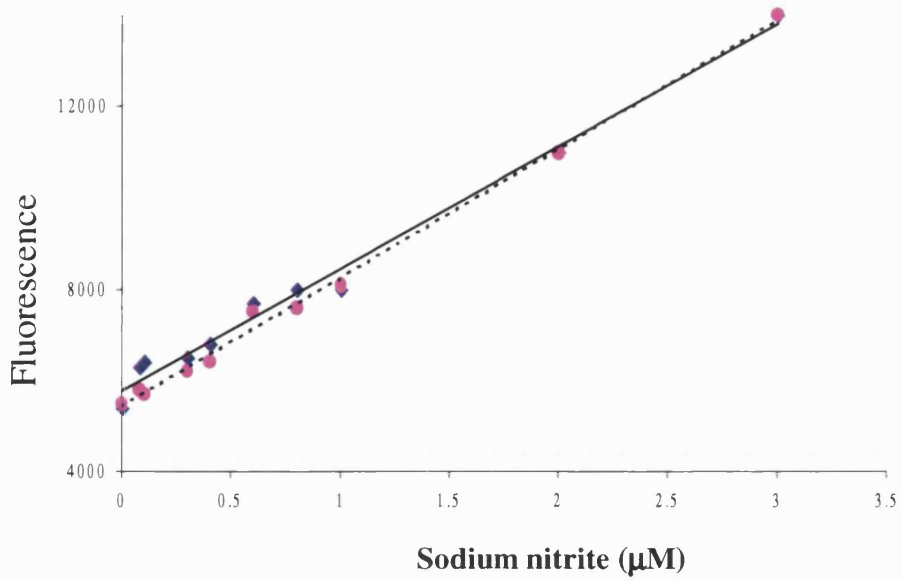


Fig. 3.7. Effect of addition of excess amounts of L-arginine on nitrite standard curve. The continuous line represents fluorescence of sodium nitrite in DMEM without L-arginine supplement and dashed line is sodium nitrite fluorescence in DMEM with L-arginine (2 mM) supplement.

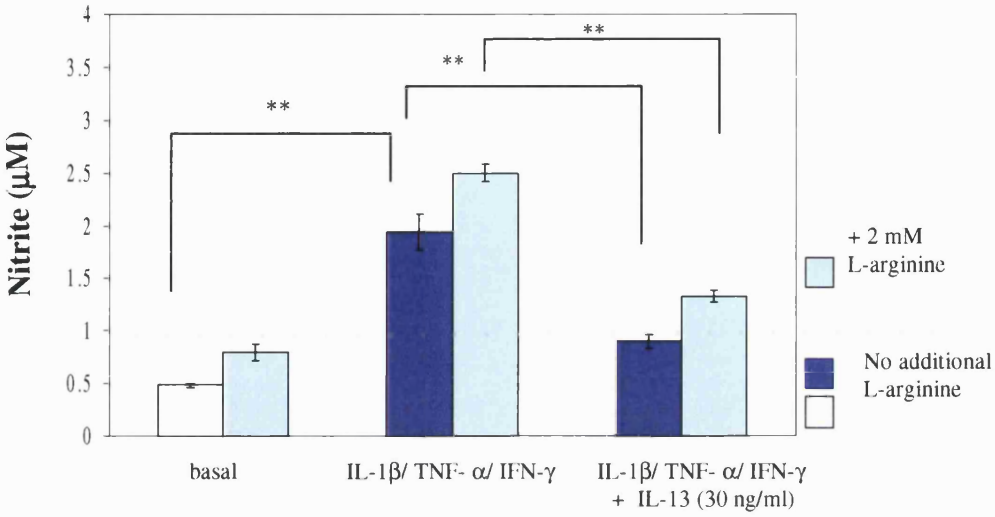


Fig. 3.8. Effect of excess extracellular L-arginine (2 mM) in DMEM medium, on nitrite production by A549 cells after treatment with cytokine mixture for 24 hours in the presence or absence of IL-13 (30 ng/ml) added 1 hour prior to cytokine stimulation. Each point is the mean ± SEM of three experiments. Significant changes in nitrite levels of cytokine stimulated A549 after IL-13 treatment with and without additional L-arginine are indicated by ** ($p< 0.001$).

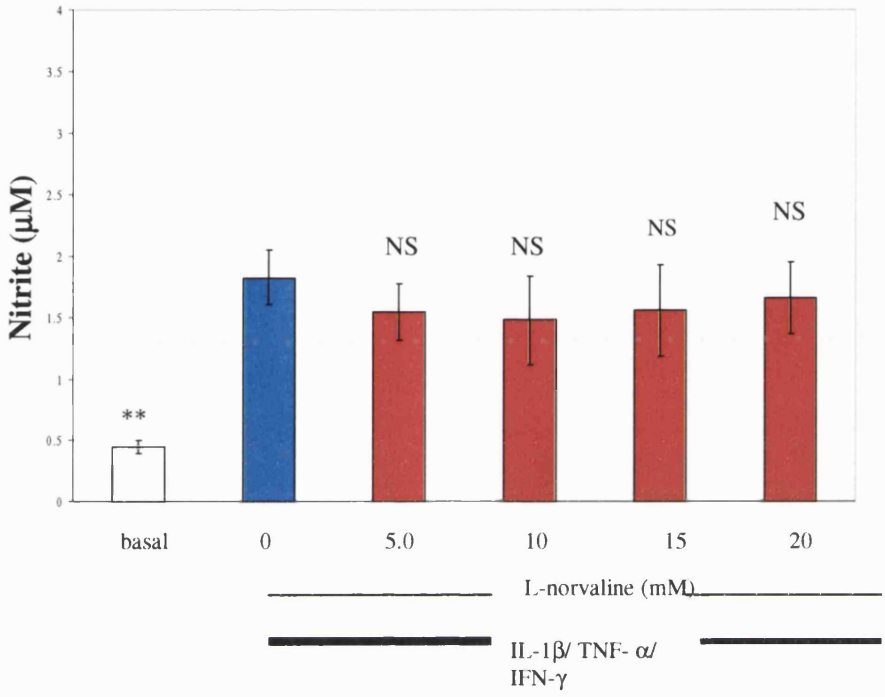


Fig. 3.9. Effect of L-norvaline (0-50 mM) on nitrite production by A549 cells after stimulation with cytokine mixture as indicated. Each point is the mean ± SEM of three experiments. NS and ** represent significant (p>0.05 and p<0.01 respectively) of L-norvaline addition compared with 0 addition to cytokine stimulated A549 .

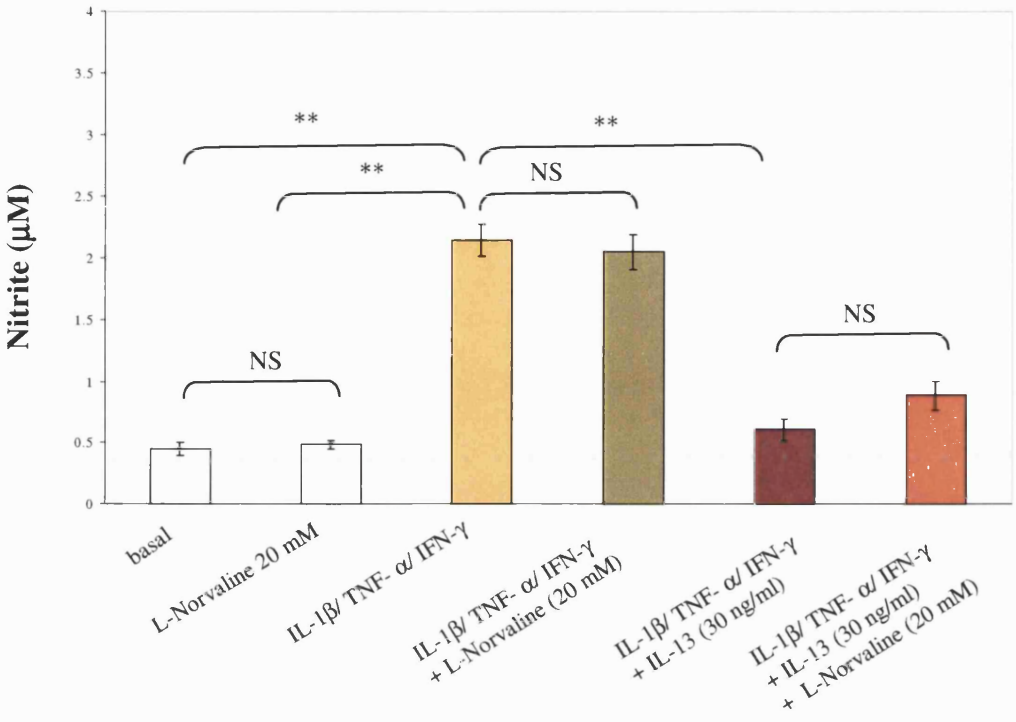


Fig. 3.10. Effect of L-norvaline (20 mM) on nitrite production by A549 cells treated with cytokines as indicated for 24 hours. Each point is the mean ± SEM of three experiments. Significance of changes in nitrite production after the addition of L-norvaline compared with basal nitrite levels represented by NS ($p>0.05$), while significant change in nitrite levels to cytokine-stimulated A549 after the IL-13 with and without the addition of L-norvaline compared with cytokine stimulatd A549. NS ($p>0.05$) and ** ($p<0.01$).

3.3 Discussion

The pulmonary epithelium, an integral component of the alveolar-capillary wall, serves multiple functions in the lung since it acts as a physical barrier between the external and internal environments. Because of the proximity of alveolar macrophages it has always been considered as a target of inflammatory cells rather than as an active participant in generation of an inflammatory response (Simon *et al.*, 1986). Recently, it has been shown that the pulmonary epithelium plays a significant role not only in the production of inflammatory cell chemoattractants (Standiford *et al.*, 1990; Palfreyman *et al.*, 1997), but also as a major and continuous cellular source of iNOS in the lung (Kobzik *et al.*, 1993; Guo *et al.,* 1995). However iNOS expression by pulmonary epithelial cell lines is present only when cell lines *in vitro* are challenged with inflammatory components (pro-inflammatory cytokines).

A frequently posed question is: what is the most suitable cell culture model to study NO derived from pulmonary epithelium? The human pulmonary epithelial cell line A549 is an alveolar type II epithelium derived from a lung adenocarcinoma derived from a 58 year old Caucasian male (Koichiro *et al.*, 1994). A549 cells are a well-established alveolar epithelial cell line and used in various investigations as a model for *in vitro* characterization of human alveolar epithelial cell functions. Robbins *et al.* (1994), Asano *et al.* (1994), Berkman *et al.* (1996), Chu *et al.* (1998) and Kwon *et al.* (2001) have demonstrated that A549 expresses iNOS and produces NO in response to pro-inflammatory cytokines. In contrast to these studies, Pechovsky *et al.* (2001) and Donnelly and Barnes (2002) showed that A549 has a different pattern of NOS isoform

expression and neither iNOS mRNA nor protein could be detected from cytokine-stimulated or non-stimulated A549.

In this study, unstimulated A549 produced a small amount of nitrite, which may be due to constitutive NOS activity, eNOS or nNOS. None of the pro-inflammatory cytokines added alone increased nitrite generation. The combination of IL-1β and IFN-γ caused an increase in nitrite generation in A549, whilst the TNF-α and IFN-γ combination was not as effective. The combination of IL-1β/TNF-α/ IFN-γ was the most effective to increase nitrite generation. It is most likely that the increased nitrite levels is due to iNOS enzyme activity because this activity was first, induced via a combination of pro-inflammatory cytokines and second, was inhibited by the iNOS by 1400W and also by aminoguanidine. These data suggest that A549 cells can be used to study nitric oxide production and regulation.

The low levels of nitrite produced in unstimulated A549 may be contributed to one or two types constitutive isoforms of NOS. Asano and associates (1994) have studied eNOS, nNOS and iNOS expression by RT-PCR in A549 cells. No evidence of eNOS (NOS3) expression was found, however, large amounts of iNOS mRNA were detected in cytokine-stimulated cells.

In a recent study to understand the transcriptional regulation of the human iNOS gene, the promoter of iNOS in A549 has been cloned, sequenced and characterized (Chu *et al.*, 1998). Consistent with the above results, their data demonstrated that iNOS mRNA was elevated in A549 cells maximally, when incubated with the cytokine combination mixture IL-1β/TNFα/IFN-γ and intermediately, when stimulated with pairwise combinations of cytokines. Single cytokines produced little effect. The synergistic

mechanism of cytokine-induced iNOS in A549 has been studied by Kwon *et al.*, (2001). They demonstrated that single cytokine stimulation led to iNOS monomer production, but only IL-1β and INF-γ exposure resulted in formation of the cofactors necessary to form the functional iNOS. The combination of cytokines activated the transcriptional factor NF-κB that augments iNOS mRNA transcription for subsequent translation.

The maximal induction of iNOS was observed only after addition of IFN-γ, indicating that IFN-γ is likely the most important component. Results from various investigations showed that IFN-γ acting in concert with pro-inflammatory cytokines potentiated that transcription of iNOS from various cell types (MacMicking *et al.*, 1997; Martin *et al.*, 1994).

The results in this work are similar to those found in cultured cells of human heptocytes (Nussler *et al.*, 1992), colon epithelial cells (Kolios *et al.*, 1995), mesangial cells (Nicolson *et al.*, 1993) and most importantly lung epithelial cells (Asano *et al.*, 1994; Robbins *et al.*, 1994; Chu *et al.*, 1998; Kwon *et al.*, 2001).

Time course studies with the combination of three cytokines demonstrated that the nitrite generation peak occurred between 24 and 48 hours after cytokine mixture stimulation. This time may be needed for protein translation or co-factor generation. In HT-29 colon epithelial cells, iNOS mRNA peaked at 24 hours and was undetectable at 72 hours (Kolios *et al.*, 1995). Nussler *et al.* (1992) and Geller *et al.* (1993) demonstrated that iNOS mRNA from human hepatocytes peaked after 8 hours after stimulation, and nitrite generation peaked at 48 hours. This gap between maximum enzyme message expression and maximum nitrite generation is required for *de novo*

synthesis of co-factors such as tetrahydrobiopterin (Stuehr and Giffith, 1992; Nussler *et al.*, 1992).

Following the discovery that L-arginine is the substrate for NO biosynthesis in most cell types, Hibbs in 1987 identified N^{ω}-substituted L-arginine analogues that suppressed NO-mediated cytotoxicity (reviewed by Whittle, 1995). Comparison studies demonstrated that N^{ω}-amino-L-arginine, followed by N^{ω}-monomethyl-L-arginine (L-NMMA) are effective inhibitors of iNOS activity in cytokine-stimulated macrophages and endothelial cells. From then, a variety of N^{ω}-substituted-L-arginine derivatives have been investigated, which basically compete with L-arginine for binding sites in the NO synthase enzymes and so inhibit their activity (Green and Nacy, 1993).

Because of the very high structural homology between iNOS and eNOS, identification of selective inhibitors has been difficult. The acetamide-containing analogues of arginine, such as aminoguanidine, which is a nucleophilic hydrazine compound, have been proposed to be highly selective for iNOS without affecting eNOS. It was found that aminoguanidine is at best only 30-fold more potent against iNOS than eNOS. Garvey *et al.* (1997), showed that N-(3-(aminomethyl)benzyl)acetamine (1400W) to be a slow, irreversible inhibitor of human iNOS. 1400W was more than 5000 and 250-fold more selective for iNOS versus both nNOS and eNOS, respectively. Its blocking reaction is an irreversible competitive inhibition with L-arginine, is NADPH-dependent and develops fairly slowly, and no significant reversal of this inhibition was observed after 2 hours. This compound shows potential as a new therapeutic agent (Alderton *et al.*, 2001; Koarai, 2000). In this study, pretreatment with 1400W and the less selective

inhibitor aminoguanidine, almost completely abolished the cytokine-induced nitrite induction from A549 cells. Thus, it is most likely that the increased nitrite levels are due to upregulation of iNOS enzyme activity

Interleukin-13 is the most recently identified cytokine to join the T helper 2 (Th2) cytokine family. It is distantly related to IL-4. It has been demonstrated to induce a variety of immunomodulatory functions on a wide range of cell types restricted to activated T lymphocytes, especially Th2 cells, mast cells, basophils, dentritic cells and natural killer (NK) cells highlighting its pleiotropic activities (Brombacher, 2000). The induction of iNOS in pro-inflammatory cytokine stimulated A549 was partly suppressed by IL-13. IL-13 produced a concentration related inhibition of nitrite accumulation induced by the optimal cytokine combination IFN-γ/TNF-α/IL-1β for 24 hours. Inhibition was maximal at the high concentration of IL-13 (30 ng/ml) added one hour prior to cytokine stimulation IFN-γ/TNF-α/IL-1β. This important inhibitory effect of IL-13 on cytokine-stimulated iNOS, was previously shown in macrophages (Liew *et al.*, 1991; Doherty *et al.*, 1993) and human pulmonary epithelium A549 cell line (Berkman *et al.*, 1996). These observations implied that the inhibitory effect of IL-13 was at the transcriptional level and suggested to be through other transcriptional factor(s), since IL-13 inhibition was reversed after addition of cycloheximide, which is a protein synthesis inhibitor. However, it was demonstrated using the human colonic epithelial cell line HT-29, that IL-13 could downregulate iNOS through the activation of phosphatidylinositol 3-kinases (PI 3-kinase) (Wright *et al.*, 1997).

Phosphatidylinositol 3-kinases are a growing subfamily of lipid kinases that catalyze the addition of a phosphate molecule to the hydroxyl group at the D3- position on the inositol ring of phosphoinositide lipids (Wymann and Pirola, 1998; Fruman and Cantley, 2002). Phosphatidylinositol (PI), the precursor of all phosphoinositides, constitutes less than 10% of all total lipids in eukaryotic cell membranes. However, less than 0.23% of the total inositol-containing lipids are phosphorylated at the 3-position, consistent with the idea that these lipids exert specific regulatory functions inside the cell, as opposed to a structural function (Rameh and Cantley, 1999).

The concept that increased PI 3-kinase activity negatively regulates iNOS has been adopted and studied by other investigators at the molecular level. For example, expression of a constitutively active class 1_A p110 subunit of PI 3-kinase attenuated iNOS promoter activity in LPS activated macrophages (Diaz-Guerra *et al.*, 1999) as well as inhibited cytokine-induced iNOS in astrocytes (Pahan *et al.*, 1999). Together, these data suggest that inhibition of PI 3-kinase activity may be significant for iNOS stimulation.

The exact mechanism underlying the regulation of iNOS expression via PI 3-kinase is not clear yet. One candidate mechanism involves the transcription factor NF-κB, which regulates the transcription of human iNOS (Guo *et al.*, 1997). IL-13 has been shown to inhibit NF- κB activation in a number of cell models including alveolar macrophages (Lentsch *et al.*, 1997; Manna and Aggarwal, 1998).

The exact signaling cascade that modulates the activity of iNOS via IL-13 is not well characterized. To investigate the relevance of PI 3-kinase to the activity of IL-13, PI 3-kinase inhibitors have been used. Nitrite levels have been partially restored when

wortmannin and LY294002 have been added prior to IL-13 and cytokine stimulation of A549.

The use of chemical inhibitors of PI 3kinase provides a fast and easy way to explore the importance of this enzyme in any cell surface receptor-mediated signaling pathway. Wortmannin (Wymann *et al.*, 1996) and LY294002 (Vlahos *et al.*, 1994) are effective selective inhibitors of PI 3-kinase that have helped to define the PI 3-kinase signaling pathways. Wortmannin is classified as a non-competitive inhibitor at nanomolar concentrations, while LY294002 competitively inhibits PI 3-kinase at micromolar concentrations (Wymann *et al.*, 1996). It was demonstrated that wortmannin and LY294002 restored partially the expression of iNOS in HT-29. Recently, wortmannin has been involved in the inhibition of PI 3-kinase in colonic epithelial cells and the induction of NF-κB independently of any effect on I κB (Wang *et al.*, 2000).

It was already established that induction of iNOS to produce high levels of NO is dependent on an adequate L-arginine supply (Moncada and Higgs, 1993; Nathan and Xie, 1994). Besides iNOS, there is another major L-arginine-consuming enzyme, arginase (Fig. 3.11), which was found to be highly active in activated murine macrophages (Granger *et al.*, 1990; Schneider and Dy, 1985). In addition, it was demonstrated that IL-13 down-regulates NO by substrate depletion through Stat-6 production of arginase in activated murine macrophages (Rutschman *et al.*, 2001). We postulated that competition between arginase and NOS, and cytokine-stimulated iNOS in particular, for their common substrate L-arginine could be a limiting factor for NO production. We predicted that if IL-13 modulates iNOS via stimulating arginase, L-arginine will be depleted and nitrite levels from cytokine-stimulated iNOS will be

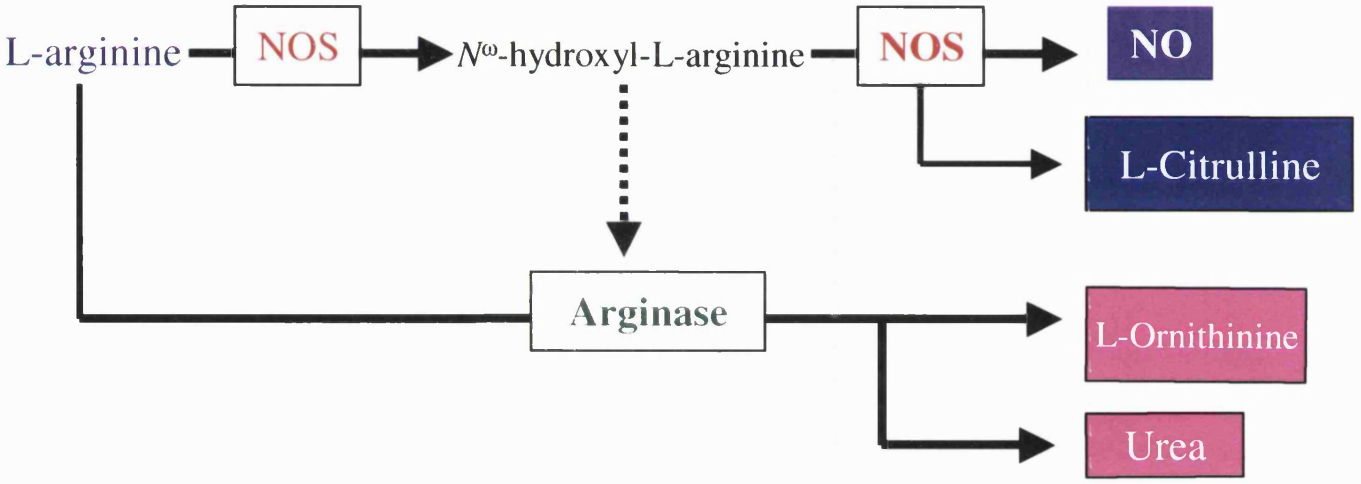


Fig. 3.11. Regulation of nitric oxide production by arginine metabolic enzymes. NOS; nitric oxide synthase, NO, nitric oxide .

reduced. Consequently, supplying excess extracellular L-arginine would restore nitrite levels, but this did not happen. IL-13 pretreatment inhibited nitrite accumulation from cytokine stimulated A549 and addition of extracellular L-arginine did not restore nitrite levels. The slight elevation of nitrite from unstimulated as well as cytokine-stimulated A549, however, may be due to cNOS or iNOS activity as abundant supply of the extracellular substrate is available for utilization.

No arginase activity could be detected in A549 cells. An arginase inhibitor, L-norvaline, was used to detect any change in cytokine-induced iNOS activity. L-norvaline is a nonmetabolizable analogue of L-valine, which has structural similarity to ornithine. Ornithine is an end product of arginase pathway and it was shown to inhibit arginase indirectly (Wu *et al.*, 1998). However the inhibitory effect of L-norvaline was demonstrated at the enzyme kinetic level, without affecting iNOS activity (Chang *et al.*, 1998). The inhibition of arginase by L-norvaline would increase substrate availability for cytokine-stimulated iNOS and more nitrite accumulation would be expected. However, L-norvaline did not cause an increase in nitrite levels from cytokine-stimulated as well as unstimulated A549. More importantly, if IL-13 modulated iNOS activity through arginase, L-norvaline and IL-13 pretreatment to stimulated A549 would restore nitrite level but again this did not occur. Accordingly, L-arginine is not a limiting factor in the stimulated iNOS and IL-13 does not modulate iNOS activity through arginase activity in the pulmonary epithelial cell line A549.

Following initial reports that NO synthesis and arginase were co-induced in macrophages, several laboratories established that iNOS might be regulated through arginase activity. Most of these studies hypothesized that substrate competition is the

key. In reality, the basis for interplay between arginase and NOS is more complex than the fact that they use a common substrate. For example, iNOS-expressing macrophages and endothelial cells can produce sufficient N^{ω}-hydroxyarginine, an intermediate in the NOS catalyzed conversion of L-arginine, to inhibit arginase activity (Fig. 3.11). Since endothelial cells in intact animals are perfused, whereas cultured cells are not, it is not clear that the former would be exposed to sufficient N^{ω}-hydroxyarginine to inhibit cellular arginase activity. In addition it must be taken into consideration that the arginine concentrations in culture media are up to ten times higher than plasma arginine concentrations, so that rates of N^{ω}-hydroxyarginine production in culture media are probably greater than *in vivo* (Muijsers *et al.*, 2001). There are two isoforms of arginase in vertebrates, both of which catalyse the conversion of arginine to ornithine and urea. They differ with regard to subcellular localization, tissue distribution, and certain enzymatic properties, reflecting the fact that they are encoded by different genes (Jenkinson *et al.*, 1996). Type I Arginase (AI), a cytosolic enzyme, is highly expressed in human liver and to a limited extent in a few other cells, like neutrophils and rat endothelial cells. In contrast, type II arginase (AII), a mitochondrial enzyme, is expressed in lower levels in kidney, brain, small intestine, mammary gland and macrophages, with little or no expression in liver. In an *in vivo* study, AI, and not AII was demonstrated to be induced by LPS in the lung and down-regulated NO from LPS stimulated iNOS (Sonoki *et al.*, 1997). As stated earlier, several studies demonstrated the co-induction of arginase and iNOS in macrophages, however, none of these studies compared the relative potency of AI and AII in regulation of NO production. It seems

that if NO levels are high both arginase isoforms are equally effective in inhibiting NO production by limiting L-arginine availability to iNOS (Que *et al.*, 2002).

In summary, A549 cells can be induced by a combination of pro-inflammatory cytokines to express iNOS. The iNOS substrate inhibitors and the anti-inflammatory cytokine IL-13 can modulate the activity of iNOS, whereas arginase has no role in iNOS regulation in A549. In conclusion, these data are consistent with the hypothesis that aberrant regulation of NO activity in CF could be due to perturbed PI 3-kinase signaling.

4. Antioxidant enzymes of *B. cepacia* strains

4.1. Catalase activity of *Burkholderia cepacia* strains

In aerobic organisms the metabolism of oxygen can result in the production of large quantities of toxic reactive intermediates such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) that readily react with various cellular components causing lethal damage. Thus oxidative stress is an inevitable consequence of aerobic life. As well, pathogens face the additional challenge of oxidative intermediates produced by neutrophils and macrophages. Upon infection, these phagocytes suddenly increase oxygen consumption and produce reactive oxygen intermediates in a process termed the respiratory stress (Brown *et al.*, 1995; Halliwell and Gutteridge, 1999). Superoxide dismutase (SOD) and catalase are well-known examples of antioxidant enzymes utilized by prokaryotes. SOD detoxifies O_2^- anions by a dismutation reaction that generates H_2O_2 and O_2, while catalase converts toxic H_2O_2 to O_2 and H_2O (Fridovich, 1978). The monofunctional catalase (HPII) is expressed in most aerobic bacteria from the *KatE* gene. Multiple catalase KatE isozymes have been observed in response to oxidative stress and growth conditions (Christman *et al.*, 1985; Loewen *et al.*, 1985a; Katsuwon and Anderson 1992; Loewen, 1996; Vattanaviboon and Mongkolsuk, 2000).

Previous work indicated that *B. cepacia* complex isolates, survive intracellularly within murine macrophages (Saini *et al.*, 1999) and human macrophages (Martin and Mohr, 2000), respiratory epithelial cell lines (Burns *et al.*, 1996) and free-living amoebae (Marolda *et al.*, 1999). The ability of *B. cepacia* strains to survive intracellularly is

mainly attributed to their ability to resist and inhibit the toxic effects of reactive oxygen species. Since *B. cepacia* isolates are known to produce catalase and SOD (Lefebre and Valvano, 2001) both enzymes are thought to protect *B. cepacia* from oxidative damage and may contribute to intracellular survival. However, intracellular survival does not appear to be a function common to all strains of *B. cepacia* complex (Martin and Mohr, 2000). In addition, Smith *et al.* (1999) observed variations in the lethal response by NO and H_2O_2 among strains of *B. cepacia.* Our hypothesis is that there may be variations in catalase (KatE) activity between strains of *B. cepacia* that may contribute to variations in susceptibility to oxidative killing. Thus, the targets were to investigate catalase (KatE) activity of four selected strains of *B. cepacia,* and to isolate and sequence the *katE* gene from JL2315 which was not killed by H_2O_2 in Smith and coworkers' study (Smith *et al.*, 1999).

The biofilm mode of bacterial growth is the preferred form of bacterial existence in nature and it has been observed that the production of catalase and SOD vary in *P. aeruginosa* when grown as biofilm when compared with planktonic culture (Hassett *et al.*, 1999a and 1999b; Bollinger *et al.*, 2001). Since *B. cepacia* cells may also grow as a biofilm in the CF lung, catalase activity was investigated from the four strains of *B. cepacia* grown as biofilm in parallel with planktonic cultures.

4.2 Results

4.2.1 Planktonic and biofilm growth phase variations of *B. cepacia* strains

The strains J2315, J2552, JL27 and JL32 were selected for this study. The strains J2315 and JL27 produced similar macroscopic cultural characteristics when grown on LB agar. Colonies of both strains were pin-point, dry, opaque and produced an extracellular brown pigment that caused discoloration of the medium. In contrast, colonies of J2552 and JL32 were mucoid, white-creamy and opaque with no pigmentation of the medium. Upon preliminary experiments of catalase activity by adding a few drops of 3% H_2O_2, both J2315 and 27 demonstrated marked evolution of gas compared with J2552 and JL 32 that produced very little gas. To study the catalase activity of these four strains in planktonic and biofilm cultures at two different growth stages, the growth characteristic of each strain was established. For planktonic cultures, preliminary experiments with LB broth cultures were performed to determine that OD_{470} values 0.3-0.5 and 3.0-5.0 corresponded to mid-logarithmic (mid-log) and early stationary phase, respectively (Fig. 4.1). The doubling time for J2315 and JL27 was 2 hours in LB broth medium, while J2552 and JL32 grew faster with a doubling time of 30 minutes.

The growth curves of the bacterial strains J2315 and JL27 forming a biofilm could be divided into a lag phase, followed by logarithmic phase after 10 hours and ended in stationary phase after 20 hours. While for the bacterial strains of J2552 and JL32, the lag phase was followed by an logarithmic phase after 5 hours and ended in stationary phase after 18 hours (Fig 4.2a and b).

This enabled investigation and comparison of the catalase activity of the four strains of *B. cepacia* at mid-log and early stationary phases of planktonic and biofilm cells.

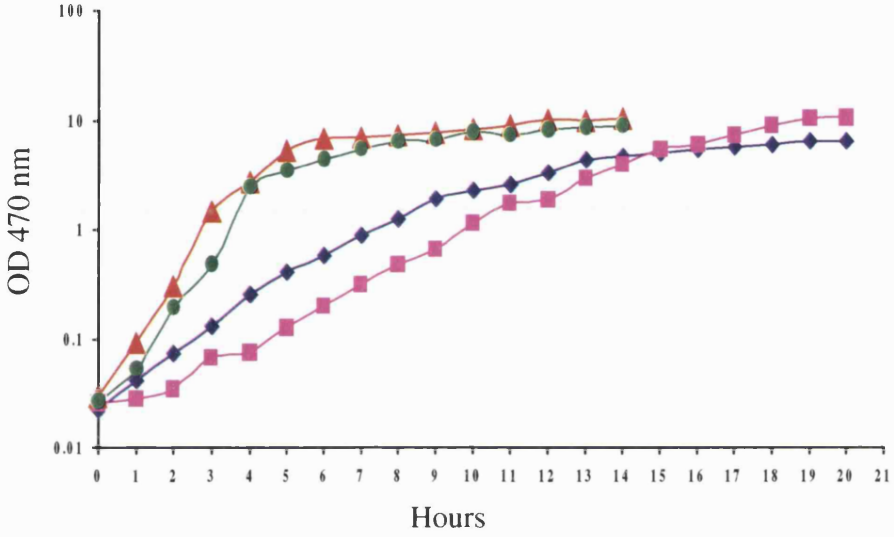


Fig 4.1. Growth of *Burkholderia cepacia* strains in planktonic culture in LB broth at 37°C.
B. cepacia (▲) J2552, (●) JL 32, (◆)JL 27 and (■) J2315 strains.

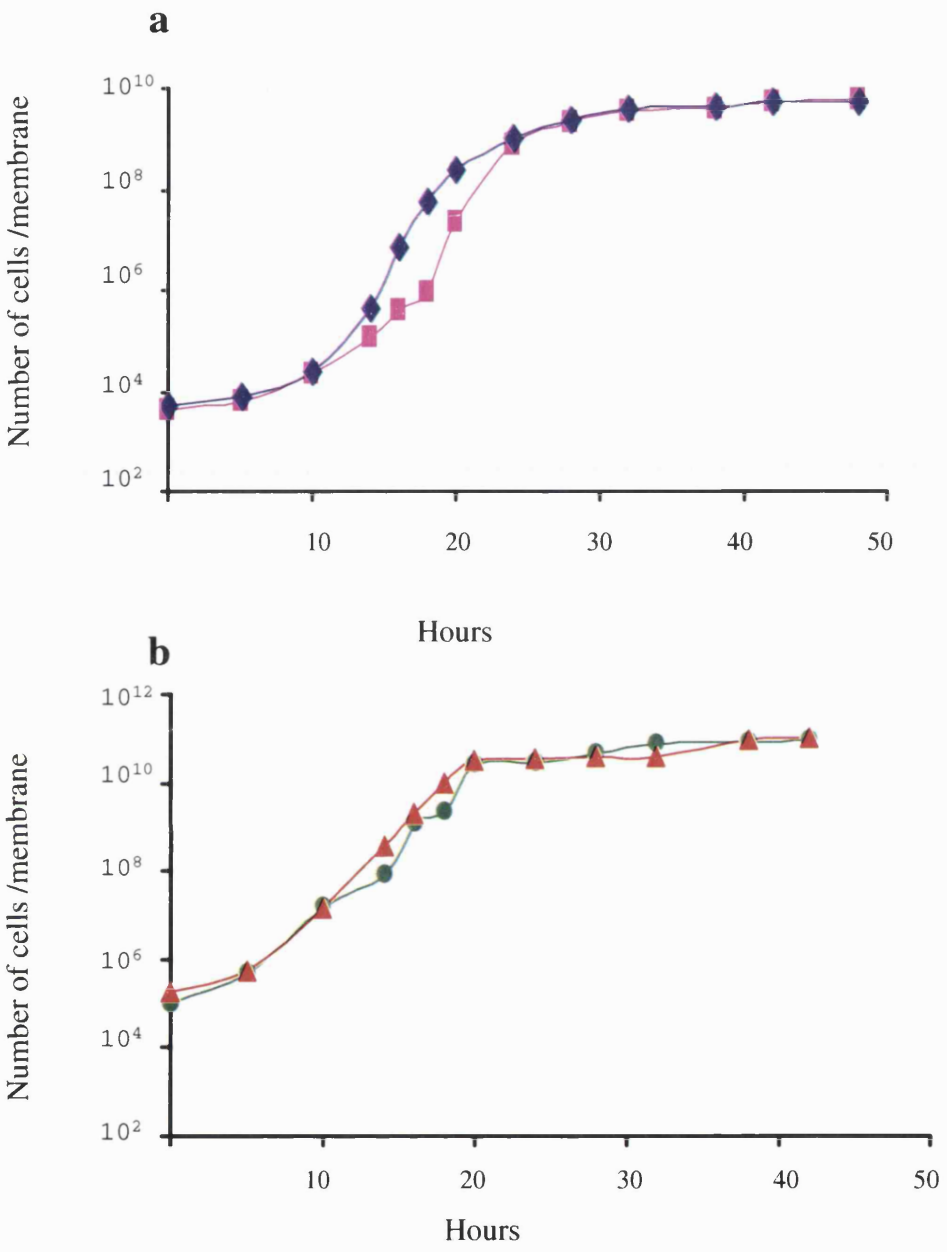


Fig. 4.2.a and b. Growth of *Burkholderia cepacia* as a biofilm on a membrane support on LB agar at 37°C.
(a) (◆)JL 27 and (■) J2315 strains, and
(b) (▲) J2552 and (●) JL 32 strains.

4.2.2. Planktonic *B. cepacia* cells versus biofilm H_2O_2 susceptibility in mid-log and early stationary phase

In many bacteria, the response to oxidative stress varies according to growth phase (Loewen *et al.*, 1985; Katsuwon and Anderson, 1992; Loewen, 1996; Vattanaviboon and Mongkolsuk, 2000). Also in recent work it was observed to be variable when cells are growing as planktonic or biofilm (Hassett *et al.*, 1999a and b; Bolliger *et al.*, 2001). The susceptibility of the four strains of *B. cepacia,* growing as biofilm or planktonic at mid-log and early stationary phase to the exposure of 5 mM H_2O_2 added extracellularly for 60 minutes was observed and results were recorded and expressed as percent surviving fraction. There was a significant reduction in surviving fraction of planktonic J2552 and JL32, but planktonic J2315 and JL27 were less susceptible. In all cases, survival increased in stationary-phase planktonic cultures when compared to survivals at mid-log phase. The planktonic J2552 and JL32 were killed after 60 minutes of exposure to H_2O_2, with more than 4 log cycle decrease in survival at mid-log and stationary phase (Fig.4.3 and Fig 4.4). The planktonic J2315 showed a high survival rate at mid-log as well as early stationary phase, with less than 1 log cycle decrease in survival at mid-log phase (Fig. 4.5). The mid-log and early stationary planktonic cells of JL27 survived after 60 minutes exposure to 5 H_2O_2 with 1 log cycle decrease in survival respectively (Fig. 4.6).

For biofilm cells, the same pattern of susceptibility for the four strains was observed as in planktonic cells. Interestingly, biofilms of all cells were more susceptible at early stationary than mid-log phase, following 5 mM H_2O_2 exposure for 60 minutes.

At early stationary phase biofilm cells of J2552 and JL32 strains were killed after 30 and 45 minutes of exposure to 5 mM H_2O_2, respectively, with a 3 log cycle decrease in survival (Fig. 4.3 and Fig 4.4). While at mid-log phase biofilm cells of both strains were killed after 45 and 60 minutes, respectively with more than a 4 log cycle decrease in survival (Fig. 4.3 and Fig. 4.4). The biofilm J2315 strain survived killing with 5 mM H_2O_2, with less than one log cycle decrease in survival at mid-log and stationary phase after 60 minutes exposure (Fig. 4.5). As well, the biofim cells of JL27 were resistant to killing with 5 mM H_2O_2, but with a one log cycle decrease in survival after 60 minutes of exposure to H_2O_2 (Fig. 4.6).

In order to compare differences in susceptibility between planktonic and biofilm cells at mid-log and early stationary phase to killing with 5 mM H_2O_2, the percent surviving fraction of J2315 and JL27 to H_2O_2 killing at 60 minutes of exposure were plotted for each strain separately. There were no significant differences in susceptibility of planktonic and biofilm cells of J2315 to 5 mM H_2O_2 at either, mid-log and early stationary phase (Fig. 4.7a). Biofilm cells of JL27 at early stationary phase were more susceptible than its planktonic counterpart ($p < 0.05$). While at mid-log phase of planktonic and biofilms there were no significant differences in susceptibility (Fig. 4.7b)

4.2.3. Catalase expression by B. cepacia planktonic and biofilm cells at mid-log and stationary phase

Catalase specific activities have been determined to correlate the H_2O_2 sensitivity of the four strains to the level of expressed catalase. This enzyme has been assayed from cell

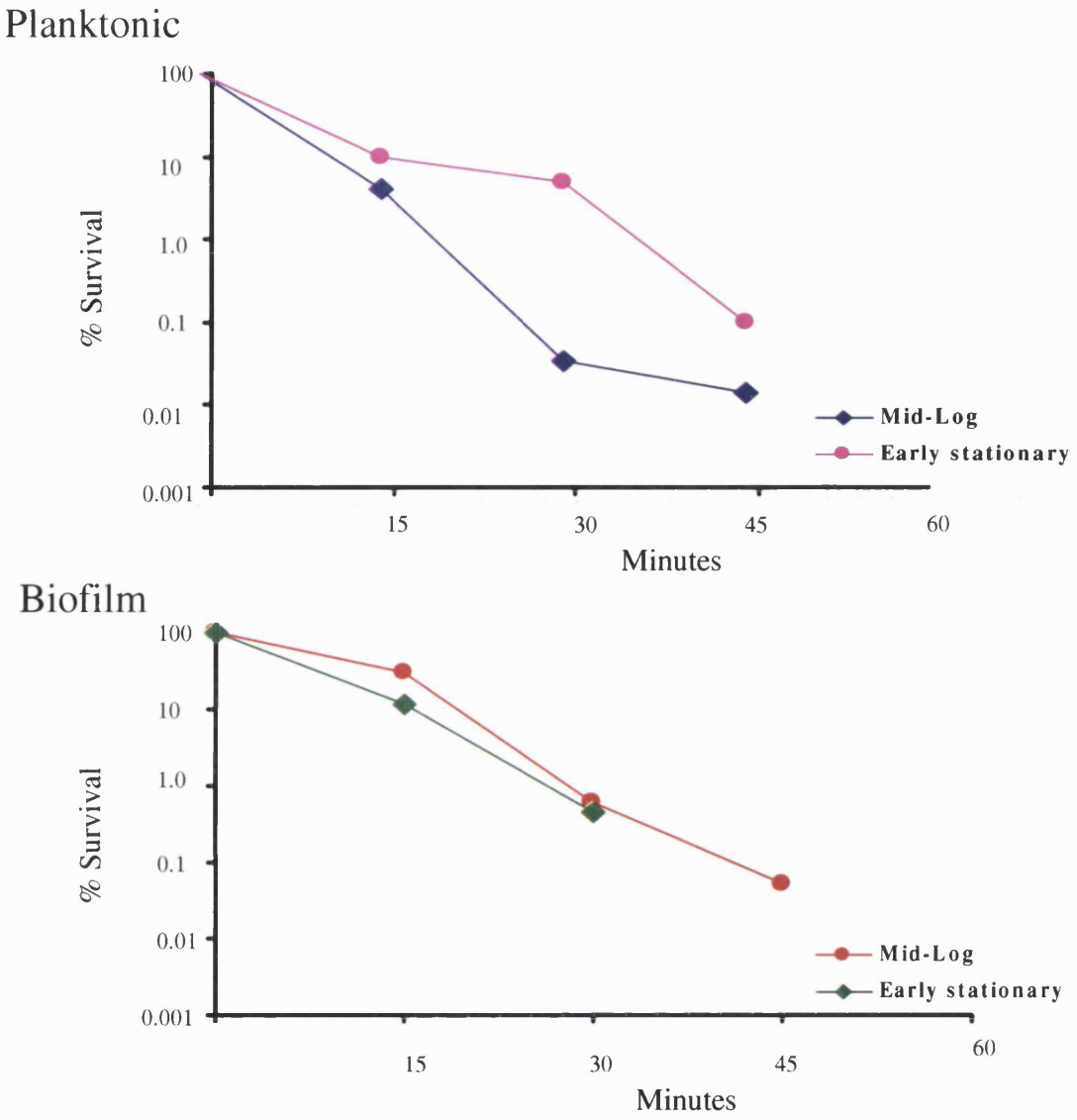


Fig 4.3. Susceptibility of planktonic and biofilm of *B. cepacia* J2552 to 5 mM H_2O_2 after growth to mid-log and early stationary phase. Samples were plated in triplicate for colony count and percentage survival was calculated relative to colony counts of untreated bacteria. Each point is the mean. SEM values were not shown because it is smaller than the symbol.

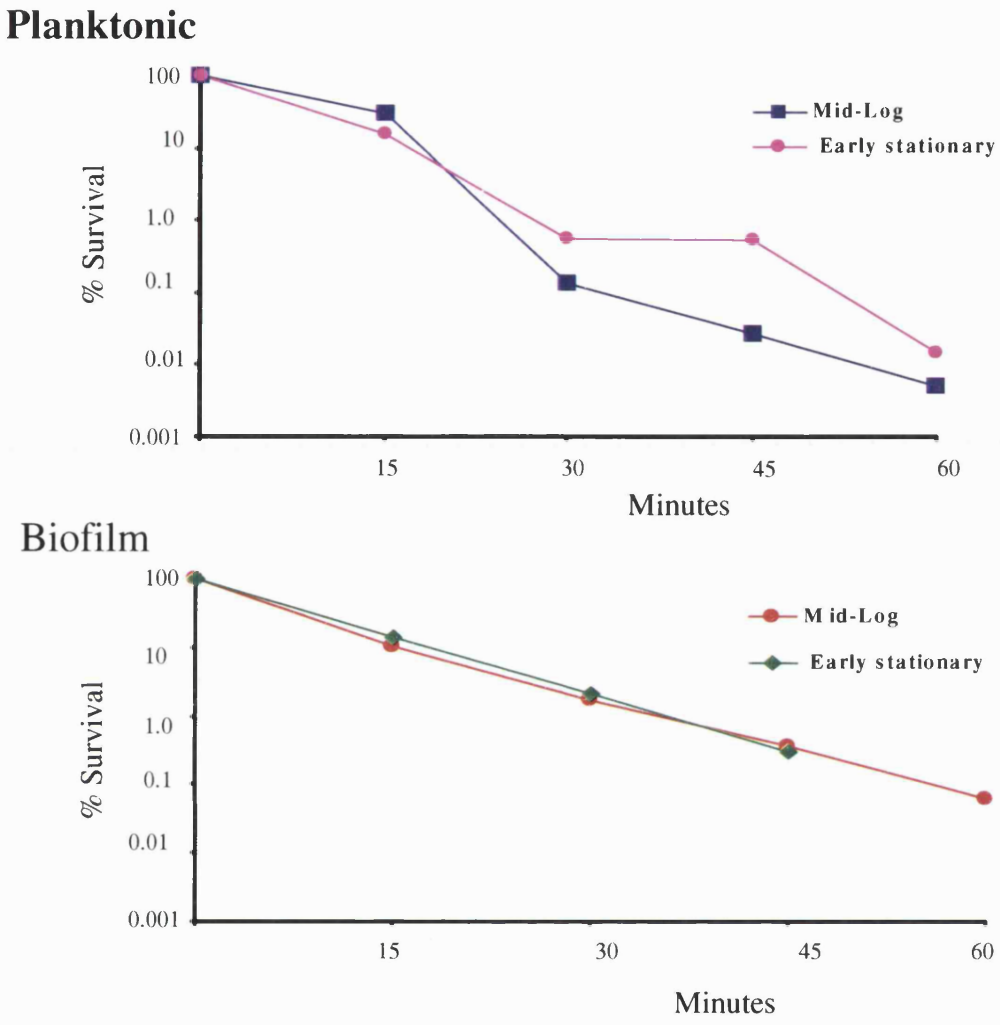


Fig 4.4. Susceptibility of planktonic and biofilm *B. cepacia* JL32 to 5 mM H_2O_2 after growth to mid-log and early stationary phase. Samples were plated in triplicate for colony count and percentage survival was calculated relative to colony counts of untreated bacteria. Each point is the mean. SEM values were not shown because it is smaller than the symbol.

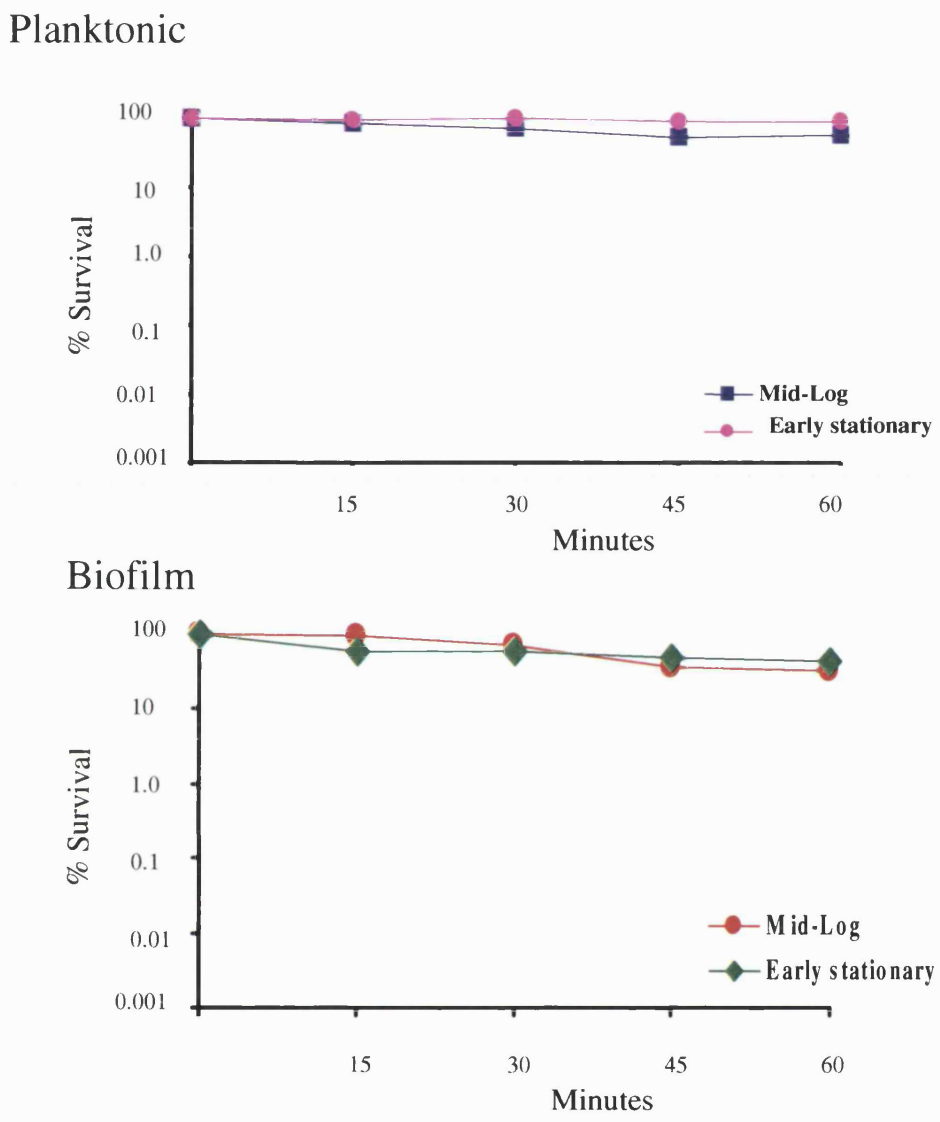


Fig 4.5. Susceptibility of planktonic and biofilm *B. cepacia* J2315 to 5 mM H_2O_2 after growth to mid-log and early stationary phase. Samples were plated in triplicate for colony count and percentage survival was calculated relative to colony counts of untreated bacteria. Each point is the mean. SE values were not shown because it is smaller than the symbol.

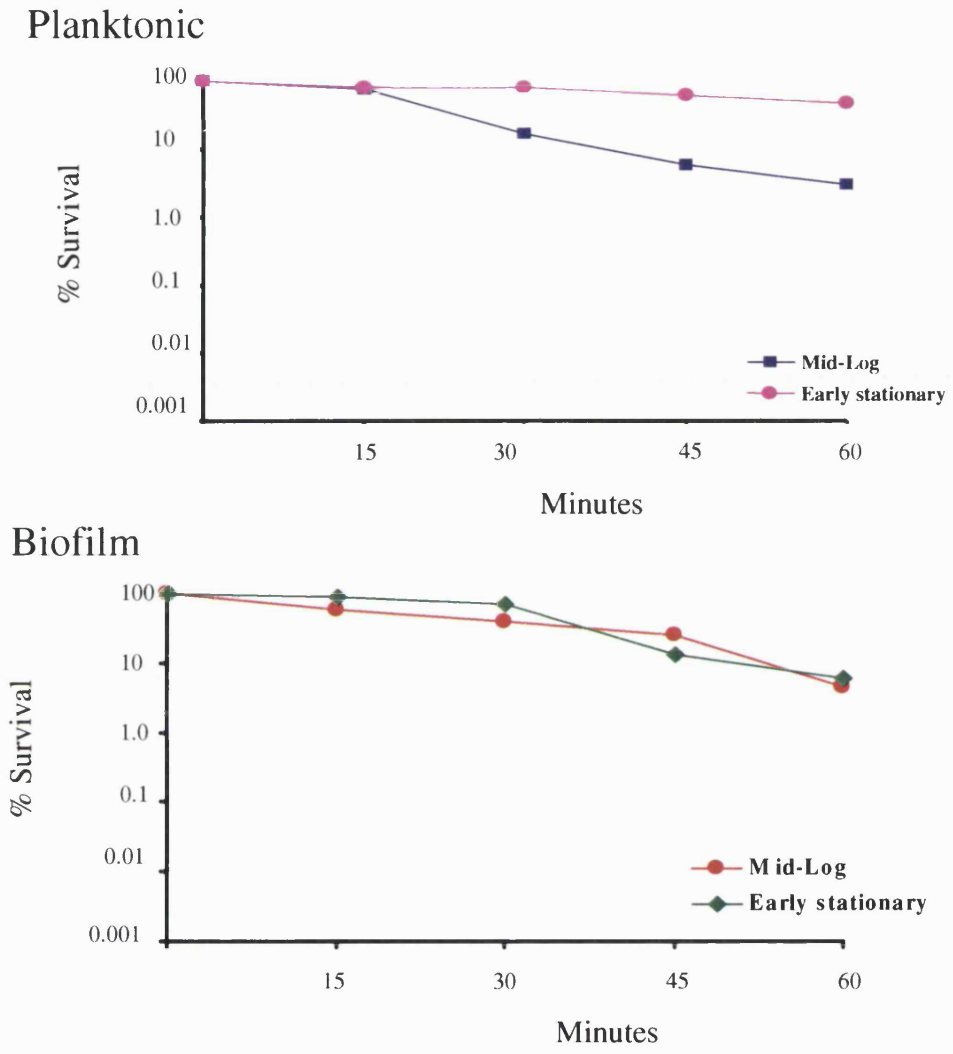


Fig 4.6. Susceptibility of planktonic and biofilm *B. cepacia* JL27 to 5 mM H_2O_2 after growth to mid-log and early Stationary phase. Samples were plated in triplicate for colony count and percentage survival was calculated relative to colony counts of untreated bacteria. Each point is the mean. SEM values were not shown because it is smaller than the symbol.

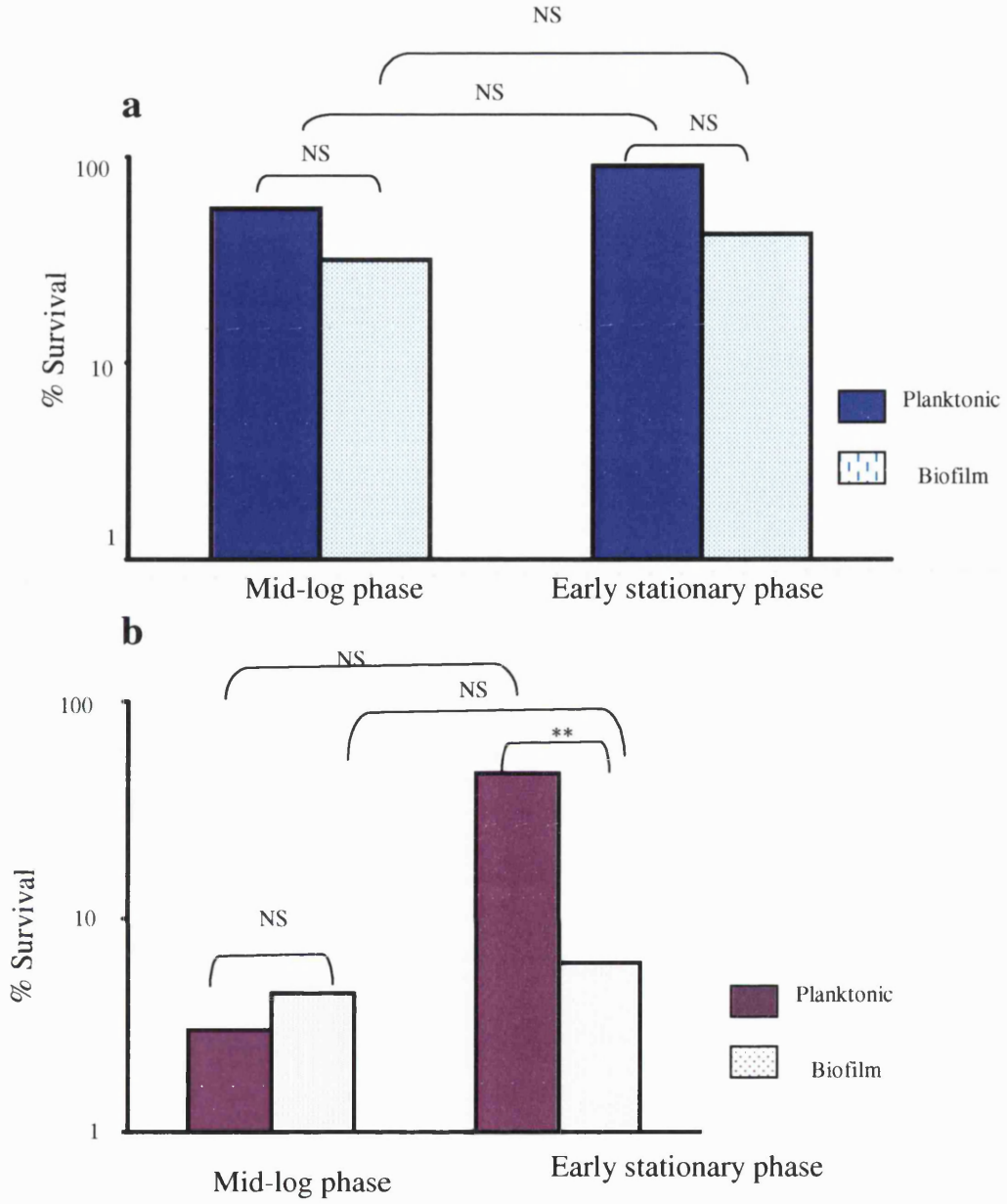


Fig. 4.7. Comparison of growth inhibition of planktonic cells and biofims of *Burkholderia cepacia* strains after 60 minutes exposure to 5 mM H_2O_2. *(a) B. cepacia* J2315 strain, and (b) *B. cepacia* JL27 strain.
Significant differences between cell survival of mid-log and stationary phase and of planktonic and biofilm cultures are indicated by NS (not significant, $p > 0.05$), and **($p < 0.05$)

lysates, not challenged with H_2O_2, from biofilm and planktonic cultures at mid-log and early stationary phase. The results of specific catalase activity were consistent with the sensitivity of the four strains upon exposure to 5 mM H_2O_2 for 60 minutes. Generally, the specific catalase activities of the planktonic cultures for the four strains were higher at stationary phase than mid-log phase On the contrary the catalase activity was reduced at the stationary phase of biofilm cultures of all four strains. The J2315 and JL27 catalase activity was higher than J2552 and JL32 that were more susceptible to 5 mM H_2O_2 for 60 minutes (Table 4.1).

In J2315 planktonic cells, catalase was expressed at mid-log and early stationary phase, with maximal expression occurring during the transition to stationary phase. However, in J2315 biofilm cells, catalase activity was 2-fold higher at mid-log phase than early stationary phase cells. In JL27 planktonic cells the moderate catalase activity at mid-log phase also increased during transition to stationary phase, but in biofilm cells catalase activity at mid-log phase was 4-fold higher than early stationary phase (Table 4.1).

In J2552 and JL32 planktonic cells, catalase activities were very low at mid-log phase with a slight increase at stationary phase, however, in J2552 and JL32 biofilm cultures catalase activities were very much lower than in planktonic cultures from both mid-log as well as stationary phase (Table 4.1).

The isozyme profiles from the planktonic and biofilm cultures were determined with cell lysates from the two stages of growth, mid-log and early stationary, by staining for catalase activity after separation on native PAGE gels (Fig. 4.8 and 4.9, respectively). It should be noted that the band intensity is not strictly proportional to the catalase activity of the band, which is a drawback this method shares with other negative stains

	Strain	mid-log		early stationary	
		Catalase activity	±SEM	Catalase activity	±SEM
Planktonic	J2315	27	±2.0	31	±1.3
	J2552	0.68	±0.02	3.2	±0.2
	JL27	10	±6.0	18	±0.1
	JL32	0.56	±1.0	5.0	±1.3
Biofilm	J2315	29	±0.7	14	±3.0
	J2552	0.05	±0.02	0.03	±0.01
	JL27	8.0	±2.0	2.0	±0.8
	JL32	0.02	±0.01	0.03	±0.02

Table 4.1. Specific catalase activity of *B. cepacia* strains.
Each reading represents the mean ± SEM of three experiments. Specific catalase activity is indicated as unites per mg protein

(Clare *et al.*, 1984; Woodbury *et al.*, 1971). J2315 and JL27 J2552 and JL32 appeared to contain only a single catalase, while JL32 demonstrated two bands of catalase activity only at mid-log phase. Enzymes with different gel mobility might be due to protein aggregates that cannot be resolved by native PAGE.

Gels of the planktonic J2315 and JL27 cells from mid-log and early stationary phase displayed one wide band of catalase at mid-log and stationary phase. For biofilm cells J2315 and JL27 produced the same single catalase band, at mid-log and early stationary phase, but with reduced intensities (Fig. 4.8 and 4.9). The catalase bands of the J2552 and JL32 biofilm started to disappear at stationary phase (Fig. 4.9).

4.2.4. Cloning of a segment of KatE gene from *B. cepacia* J2315 and J2552 strains

As *B. cepacia* strain J2315 demonstrated a phenotype characteristic of high constitutive catalase activity, while strain J2552 showed low catalase activity, a genetic approach was employed to clone and characterize the catalase gene *katE* from these two organisms. The strategy was to use PCR primers directed against conserved regions of the catalase gene to amplify an internal fragment of the catalase gene. To accomplish this, advantage was taken of the high homology between regions of *katE* of *Escherichia coli* with catalase from different phyla (von Ossowski *et al.*, 1991). Using the alignment of the primary structures of the KatE (HPII) proteins of *E. coli* and *P. aeruginosa* as a template (Fig. 4.10), degenerate oligonucleotides derived from the regions of homology between *E. coli* and *P. aeruginosa katE* gene were designed with a bias toward the high G+C codon usage of *P. aeruginosa* (West and Iglewski, 1988).

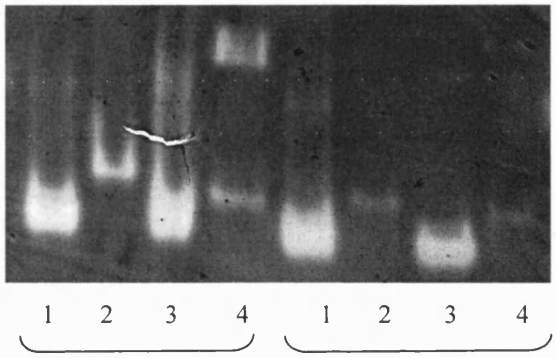


Fig. 4.8. Detection of catalase activities in planktonic cell-free lysates from *B. cepacia* strains.
Extracts were from mid-log and early stationary phase and analysed by native PAGE followed by staining described in **Methods**.(1)J2315, (2) J2552, (3) JL27, and (4) JL32.

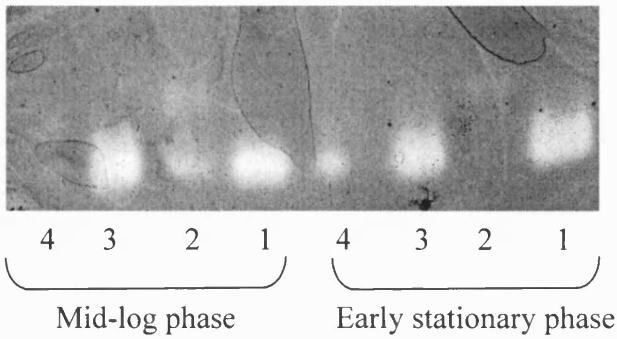


Fig 4.9. Detection of catalase activities in biofilm cell-free lysates from *B. cepacia* strains.
Extracts were from mid-log and early stationary phase and analysed by native PAGE followed by staining described in **Methods**. (1) J2315, (2) JL32, (3) JL27, and (4) J2552.

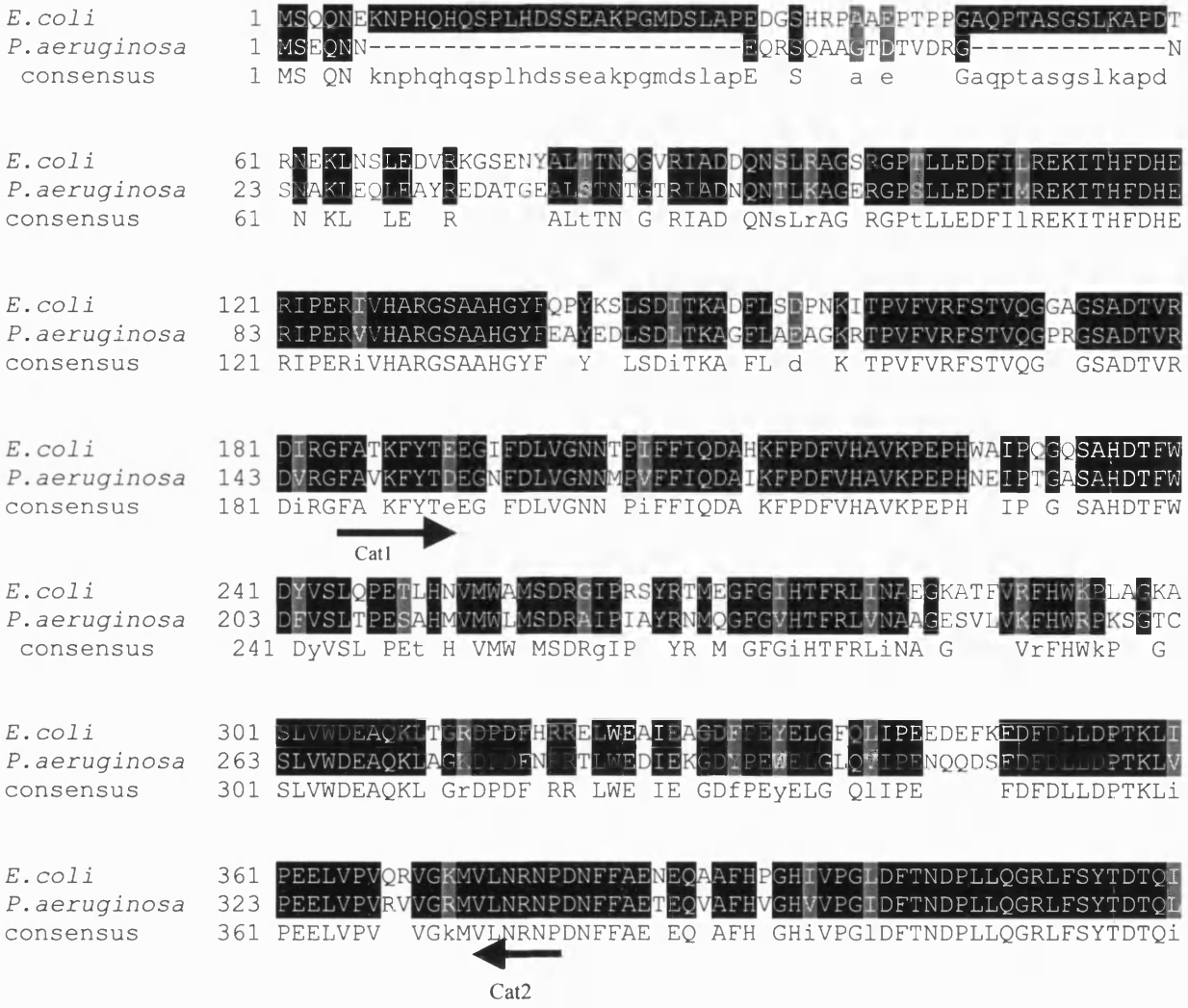
4.2.5. Comparison of amino acid sequence between *E. coli* and *P. aeruginosa* catalases to develop degenerative oligonucleotides

Comparison of the deduced amino acid sequence of the catalase HPII subunit from *E. coli* and *P. aeruginosa* revealed significant similarity between portions of HPII. The homologous sequence is presented in Fig 4.10. Identity and similarity percentages were obtained by pair-wise comparison of amino acid residues using the Blast facility provided on the NCBI website (http://www.ncbi.nlm.nih.gov) which revealed 58% identities and 70% similarities. The sites of homology were reverse transcribed into nucleotide sequence, using codon usage data (West and Iglewski, 1988) to design two degenerate oligonucleotides.

Genomic DNA from *B. cepacia* J2315 and J2552 were prepared as described in **Methods 2.4.2**. To amplify the *katE* gene in two strains of *B. cepacia* the following degenerate oligonucleotide (primer) pair was used: cat1 (5'-TTCGC **G/C** A **G/C** C **G/C** AAGTTCTACAC) and cat2 (5'-TT **G/C** CGGTT **G/C** AG **G/C** ACCAT). These primers predicted to bind 550 bp apart, and indeed 550 bp fragments were amplified for J2315 and J2552 (Fig. 4.11). The amplimers were designated as *cat21* and *cat22*.

4.2.6. Cloning of 550 bp of katE gene in pGEM-T vector

In order to characterise the *katE* gene, *cat21* and *cat22* were cloned, amplified and sequenced. The gel-purified 550 bp amplimers were cloned into the multiple cloning site of the plasmid vector pGEM-T to produce pGEM-cat:21 and pGEM-cat:22, which


E.coli 1 MSQQNEKNPHQHQSPLHDSSEAKPGMDSLAPEDGSHRPAAEPTPPGAQPTASGSLKAPDT
P.aeruginosa 1 MSEQNN----------EQRSQAAGTDTVDRG----------N
consensus 1 MS QN knphqhqsplhdsseakpgmdslapE S a e Gaqptasgslkapd
E.coli 61 RNEKLNSLEDVRKGSENYALTTNQGVRIADDQNSLRAGSRGPTLLEDFILREKITHFDHE
P.aeruginosa 23 SNAKLEQLFAYREDATGEALSTNTGTRIADNQNTLKAGERGPSLLEDFIMREKITHFDHE
consensus 61 N KL LE R ALtTN G RIAD QNsLrAG RGPtLLEDFIlREKITHFDHE
E.coli 121 RIPERIVHARGSAAHGYFQPYKSLSDITKADFLSDPNKITPVFVRFSTVQGGAGSADTVR
P.aeruginosa 83 RIPERVVHARGSAAHGYFEAYEDLSDLTKAGFLAEAGKRTPVFVRFSTVQGPRGSADTVR
consensus 121 RIPERiVHARGSAAHGYF Y LSDiTKA FL d K TPVFVRFSTVQG GSADTVR
E.coli 181 DIRGFATKFYTEEGIFDLVGNNTPIFFIQDAHKFPDFVHAVKPEPHWAIPQGQSAHDTFW
P.aeruginosa 143 DVRGFAVKFYTDEGNFDLVGNNMPVFFIQDAIKFPDFVHAVKPEPHNEIPTGASAHDTFW
consensus 181 DiRGFA KFYTeEG FDLVGNN PiFFIQDA KFPDFVHAVKPEPH IP G SAHDTFW
Cat1
E.coli 241 DYVSLQPETLHNVMWAMSDRGIPRSYRTMEGFGIHTFRLINAEGKATFVRFHWKPLAGKA
P.aeruginosa 203 DFVSLTPESAHMVMWLMSDRAIPIAYRNMQGFGVHTFRLVNAAGESVLVKFHWRPKSGTC
consensus 241 DyVSL PEt H VMW MSDRgIP YR M GFGiHTFRLiNA G VrFHWkP G
E.coli 301 SLVWDEAQKLTGRDPDFHRRELWEAIEAGDFPEYELGFQLIPEEDEFKFDFDLLDPTKLI
P.aeruginosa 263 SLVWDEAQKLAGRDPDFNQRTLWEDIEKGDIPEVELGLQVIPENQQDSFDFDLLDPTKLV
consensus 301 SLVWDEAQKL GrDPDF RR LWE IE GDfPEyELG QlIPE FDFDLLDPTKLi
E.coli 361 PEELVPVQRVGKMVLNRNPDNFFAENEQAAFHPGHIVPGLDFTNDPLLQGRLFSYTDTQI
P.aeruginosa 323 PEELVPVRVVGRMVLNRNPDNFFAETEQVAFHVGHVVPGIDFTNDPLLQGRLFSYTDTQL
consensus 361 PEELVPV VGkMVLNRNPDNFFAE EQ AFH GHiVPGlDFTNDPLLQGRLFSYTDTQi
Cat2

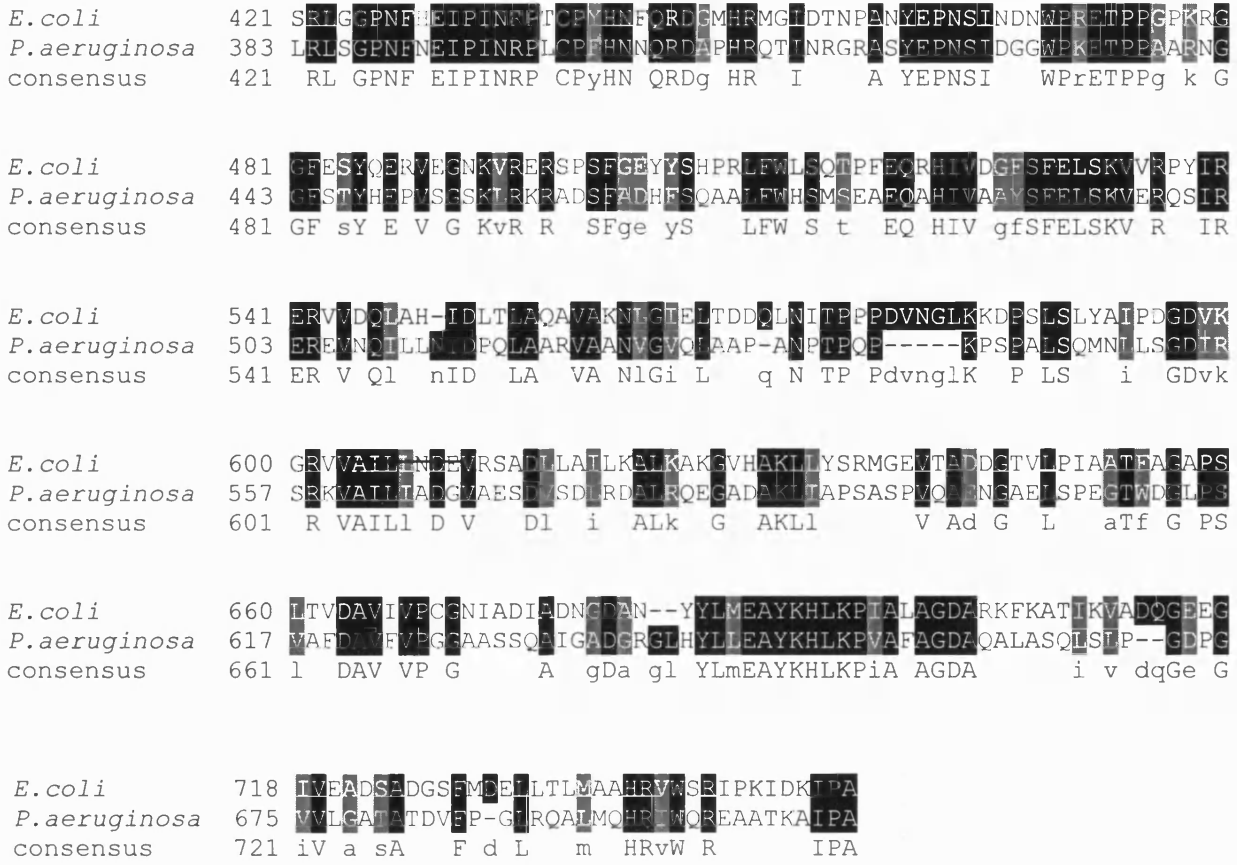


Fig. 4.10. Box-shade ClustalW analysis of HPII catalase homologous sequence of *E. coli* and *P. aeruginosa*. Dashed lines represent gaps introduced to optimise alignments. Underlined amino acid sequences denoted sites of degenerative oligonucleotides cat1 and cat2.

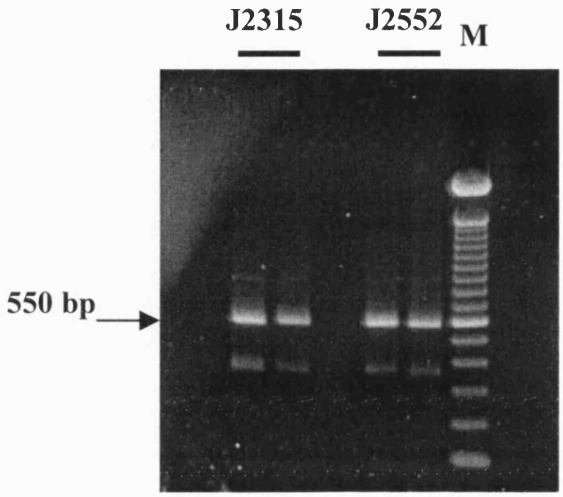


Fig 4.11. Agarose gel analysis of PCR products *B. cepacia* J2315 and J2552. Lane M is 100 bp DNA marker.

were then transformed into *E. coli* JM109 by heat shock. Transformants were recovered on selective LB agar containing 100 μg/ml ampicillin and those containing the recombinant plasmid were identified by blue-white colour selection. Successful cloning of an insert in the pGEM-T vector interrupts the coding sequence of the β-galactosidase, thus recombinant clones that contain 550 bp PCR fragment (*cat21* or *cat22*) produced white colonies.

4.2.7. Restriction enzyme mapping of pGEM-cat:21 and pGEM-cat:22 DNA

The pGEM-T contains multiple restriction sites within the multiple cloning region. These restriction sites allow for the linearisation of the recombinant plasmid. Alternatively, a double-digestion may be used to release the insert.

The pGEM-cat:21 and pGEM-cat:22 plasmids were purified as described in **Methods** 2.4.11. Plasmids were digested with *Apa* I and *Pst* I separately and in combination. The single digests linearized both pGEM-cat:21 and pGEM-cat:22 (Fig 4.12 and 4.13, respectively) and the digests were of the expected molecular weight (3.55 Kb). The double digests released 550 bp, *cat21* and *cat22* inserts from both of the pGEM-cat:21 and pGEM-cat:22 (Fig. 4.12 and 4.13, respectively).

4.2.8. Sequence analysis of the 550 bp insert cat21

The cloned 550 bp fragment in pGEM-cat21 was amplified by PCR using the vector primer pair T7 and SP6 RNA polymerase promoters flanking the multiple cloning region within the α-peptide coding region of β-galactosidase. The amplified *cat21*

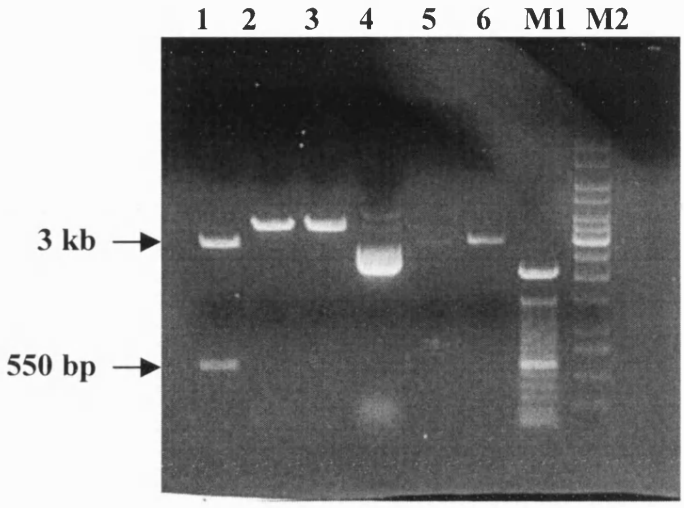


Fig. 4.12. Agarose gel analysis of restriction enzyme digests of pGEM cat:21. Lane 1 double digest with *Pst* I and *Apa* I, lane 2 single digest with *Pst* I, lane 3 single digest with *Apa* I, lane 4 undigested pGEM cat:21, lane 5 undigested pGEM-T vector, lane 6 digested pGEM-T vector with *Pst* I, lane M1 100 bp DNA marker, and lane M2 3kb DNA marker.

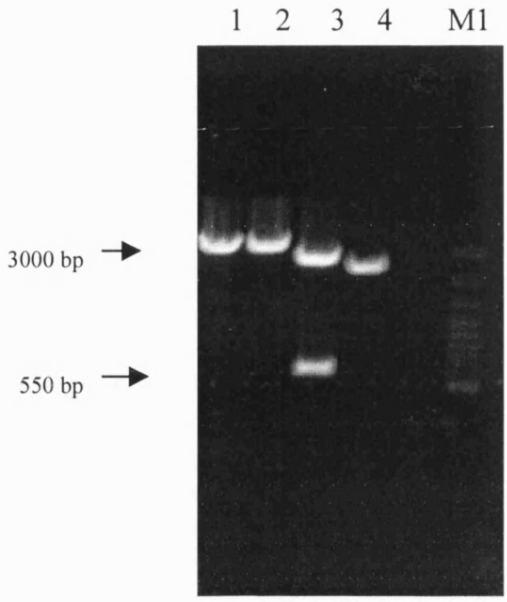


Fig. 4.13. Agarose gel analysis of restriction enzyme digests of pGEM cat:22. Lane 1 single digest with *Pst* I, lane 2 single digest with *Apa* I, lane 3 double digest with *Pst* I and *Apa* I, lane 4 undigested pGEM cat:22, and lane M 100 bp DNA marker.

fragment from pGEM-cat:21 was sequenced at the Department of Biology and Biochemistry University of Bath, using an automated DNA sequencing with d-Rhodamine terminator dye chemistry and FS *Taq* polymerase (PE Applied Biosystems). Figure 4.14 shows the alignment of the 550 bp amino acid sequence of HPII of *B. cepacia* J2315 strain with putative HPII amino acid sequence of *E. coli* and *P. aeruginosa*, which were used to design the degenerate primers.

BLAST analysis revealed that the primary sequence of catalase of *B. cepacia* J2315 is 44% identical (58% similarity) and 44% identical (55% similarity) to the putative catalase sequences of *E. coli* and *P. aeruginosa,* respectively. However, BLAST analysis to other related bacteria revealed 90% identical (90% similarity) to catalase from *P. fluorescens*, 83% identical (88% similarity) to the catK *P. aeroginosa* PAO1 strain, 81% identical (87% similarity) to the catalase from *Xanthomonas campestris*, 85% identical (77% similarity) to the catalase from *Vibrio cholerae* and 58% identical (72% similarity) to the catalase from *Bacillus anthracis*.

4.3. Methionine sulfoxide reductase (MsrA) in *B. cepacia* strains

The methionine sulfoxide reductase (MsrA) is a ubiquitous antioxidant enzyme found in a wide variety of organisms that has the ability to reverse the inactivation of many proteins due to the oxidation of critical methionine residues during oxidative stress. MsrA catalyses the reduction of methionine sulfoxide [Met(O)] residues to methionine. The oxidation of a specific methionine residue may occur by the activity of hydrogen peroxide, hydroxyl radicals, superoxide anions (Brot *et al.*, 1981) and peroxynitrite

(St.John *et al.*, 2001). In many cases, the oxidation of a specific methionine residue leads to the loss of biological activity of certain proteins required for cell viability. Thus the ability to reduce Met(O) residues in proteins is important for the survival of *E. coli* (Moskovitz *et al.*, 1995), yeast (Moskovitz *et al.*, 1997) and *Mycobacterium tuberculosis* (St. John *et al.*, 2001).

In vitro experiments revealed that the *msrA* gene is also in the class of genes that are expressed in a growth-dependent manner, since Moskovitz *et al.* (1995) showed that the synthesis of MsrA protein increased about threefold during the transition of *E. coli* cells to stationary phase.

So the hypothesis was whether such a repair system exists in *B. cepacia* strains in parallel to catalase to protect themselves from the toxic effect of reactive oxygen intermediates.

4.4 Results

4.4.1 Methionine sulfoxide reductase A, activity assay from planktonic *B. cepacia* strains

The pellets from planktonic cells of the mid-log and stationary phase of *B. cepacia* strains J2315, J2552, JL27 and JL32 were assayed for MsrA activity at the Weil Medical college of Cornell University, New York. No MsrA activity was detected at either mid-log nor at stationary phase from the four strains of *B. cepacia.* The substrate *N*-acetyl-L-[^{3}H] methionine sulfoxide was not reduced in the presence of 15 mM DTT or a thioredoxin regenerating system, indicated by the lack of radioactive product *N*-Ac-L-[^{3}H] methionine.

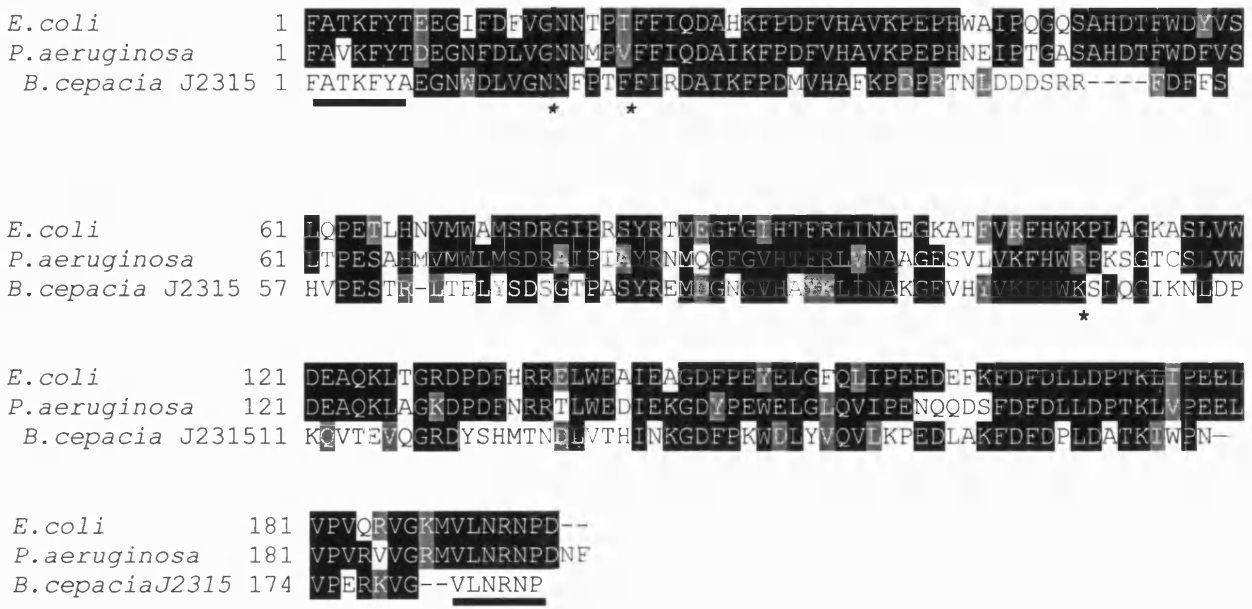


Fig.4.14. Box-shaded ClustalW analysis of HPII catalase seqment isolated from *B. cepacia* J2315 strain with homologous sequence of *E. coli* and *P. aeruginosa*. Dashes represent gaps introduce to optimise alignments. conserved residues are indicated by (*). Underlined amino acid sequences denote sites of degenerative oligonucleotides.

4.5 Discussion

Oxidative stress can be functionally defined as an excess of prooxidants in the living cell. Active oxygen molecules have been shown to cause damage to DNA, RNA, proteins and lipids as discussed previously. Active oxygen intermediates are produced as an inescapable by-product of normal aerobic metabolism, and their production is further enhanced by exposure to certain environments or by dietary or disease conditions. Under certain conditions the concentration of reactive oxygen species rises to a level that overwhelms the basal level of scavenging capacity of the cell, giving rise to an oxidative stress condition. Bacteria respond to a variety of stresses and undergo derepression of a set of globally regulated genes (Gottesman, 1984). Two of the well known responses to oxidative stress are the peroxide stress response and the superoxide stress response, that are induced under increased influx of H_2O_2 or the superoxide radical O_2^-, respectively (Farr and Kogoma, 1991). Interestingly, these two oxidative stress responses are different, since a large volume of evidence has accumulated which indicates that the peroxide and superoxide stress responses are distinct. For example, cells pretreated with H_2O_2 and cells preinduced with O_2^- generators do not develop cross-resistance (Farr *et al.*, 1985). Similarly, H_2O_2-pretreated cells reactivate H_2O_2 -damaged phage but not O_2^- -damaged phage, whereas O_2^- -pretreated cells reactivate O_2^- -damage phage but not H_2O_2 -damaged phage (Farr and Kogoma, 1991). For the most part, proteins induced with H_2O_2 are different from those induced under O_2^- generating conditions (Loewen *et al.*, 1981). In addition, it was demonstrated lately that other types of stress, such as heat shock and starvation, could also induce some proteins

that are induced by oxidative stress (Farr and Kogoma, 1991). *In vivo*, oxidative stress can be brought about by addition of H_2O_2 or superoxide anion generators, such as paraquat (Farr and Kogoma, 1991) and a mixture of hypoxanthine and xanthine oxidase (Smith *et al.*, 1999).

Genetic mapping studies revealed the presence of two independent species of catalase in *Escherichia coli*, the loci *katE* (Loewen, 1984) and *KatF* (Loewen and Triggs, 1984) affect the synthesis of an apparently monofunctional catalase which was characterized as hydroperoxidase II (HPII), while the locus *katG* affects the synthesis of the bifunctional iso-enzyme pair labeled HPI-A and –B that possess both catalase and peroxidase activity and generally called SOD. As a member of the LysR family of autoregulators, OxyR responds to the increasing oxidant levels inside the cells and activates the transcription of *katG* along with several other H_2O_2 inducible genes (Storz and Targalia, 1992). In contrast, synthesis of the HPII catalase is not induced by H_2O_2, but is dependent on RpoS, a stationary phase-specific sigma factor that is itself subject to complex transcriptional and post-transcriptional controls (Loewen and Triggs, 1984; Mulvey *et al.*, 1988; Schelhorn and Hassan, 1988; Fang *et al.*, 1992; Mulvey *et al.*, 1990; Loewen and Hengge-Aronis, 1994; Loewen, 1996; Robbe-Saule *et al.*, 2001).

Oxidative stress is strongly implicated in the CF lung where the inflammatory response is dominated by neutrophils, however, *B. cepacia* strains isolated from CF patients, especially those of genomovar III, are strong catalase and SOD producers (Lefebre and Valvano, 2001). This indicates that *B. cepacia* resistance to oxidative damage may play a role in the infectivity and persistence of this opportunistic pathogen in the CF lung. Infection of CF airways with *B. cepacia* is associated with a worse prognosis than

infection with *P. aeruginosa* (Hutchison and Govan, 1999). Moreover, differential pathogenicity among strains of *B. cepacia* has been observed in CF patients, although the basis of this differential pathogenicity amongst genomovars is unknown.

Smith *et al.*, (1999) and Pacelli *et al.*, (1995) showed that NO potentiated toxicity O_2^--mediated killing of *B. cepacia* and *E. coli*, respectively, and this killing was abolished by catalase but not SOD. Exogenously added SOD did not show any protective role in *B. cepacia* (Smith *et al.*, 1999). These data suggest that catalase, but not SOD, is responsible for the resistance of *B. cepacia* to oxidative killing *in vitro*. Moreover, some strains of *B. cepacia* showed resistance to 5 mM of H_2O_2 while others were more susceptible (Smith *et al.*, 1999). We hypothesized that since catalase activity is likely important for the virulence and pathogenicity *B. cepacia* in the CF lung, and that there are differences in pathogenicity among different strains of the *B. cepacia*, it may be possible that this is due to differences in the expression of expressed catalase levels and/or to differences in its activity.

Till this date, the catalase enzyme in *B. cepacia* has not been systematically characterized, except for the recent study of Lefebre and Valvano, (2001). In this study four strains of *B. cepacia* were selected to investigate their catalase expression and activity, which may contribute to bacterial survival and persistence in infection of the CF lung and airway. In addition the survival rate of the four strains as determined following challenge with H_2O_2 at a concentration equal or higher than those normally found *in vivo*. The strains used in this study were: *B. cepacia* strain J2315 which is a highly virulent strain belonging to genomovar III, highly transmissible and has been associated with cepacia syndrome, *B. cepcia* strain J2552, which is an environmental

isolate that belongs to genomovar I, *B. cepacia* strain JL 32 which is a CGD isolate and *B. cepacia* strain JL27 that belonges to genomovar III (Smith *et al.*, 1999). In order to gain a better understanding for the role of catalase in resistance of *B. cepacia* to H_2O_2 during infection to CF lung, catalase activity and expression were investigated in the biofilm model of bacterial cultures on the surface a filter membrane and compared with planktonic cultures. Bacterial biofilms not only exist widely in nature (Costerton *et al.*, 1995), but also in clinical settings, especially in patients with CF (Gilbert and Brown, 1995), where they show different physiological properties in their response to environmental influence compared with their planktonic models. Previous studies revealed marked changes in virulence factor production in *B. cepacia*, when grown under nutrient limitations in a chemostat model resembling the biofilm mode of growth (McKenney and Allison, 1995). Also it was demonstrated that there is a marked difference in the oxidative response in the biofilm of *P. aeruginosa* (Hassett *et al.*, 1999a and 1999b) and *Nitrosomonas europaea* (Wood and Sørensen, 2001) relative to planktonic growth.

B. cepacia J2315 strain possessed a single catalase, as revealed by electrophoretic analysis, which was expressed at mid-log and at early-stationary phase in planktonic as well as in biofilm models. In contrast, catalase activity was strongly growth-dependent in planktonic *B. cepacia* JL27, which also possessed one catalase, and its activity increased as the planktonic cells went from exponential phase to early stationary phase. In spite of lack difference of band intensities in native gels, the highest catalase specific activity from both stains, J2315 and J2552, were obtained in early stationary phase of planktonic cells. The electrophoretic analysis of J2315 and JL27, with only one catalase

class, matched the catalase profile detected from strains *B. cepacia* genomovar III in a recent study of Leferbe and Valvano, (2001) where also one catalase class was detected from genomovar III. While closely related species such as *P. aeruginosa*, *P. putida*, *P. syringae* and *Xanthomonas sp.* possess multiple catalase isozymes (Brown *et al.*, 1995; Katsuwon and Anderson, 1992; Mongkolsuk *et al.*, 1996).

The *B. cepacia* stains J2552 and JL32 possessed also one electrophoretotype catalase, at stationary phase, while at mid-log phase, only JL32 showed another band of catalase isozymes, one of which might correspond to a larger protein aggregate. However, the catalase specific activities were less than those from J2315 and J2552, but increased in planktonic cells entering stationary phase. In addition, the resistance to superoxide killing of the planktonic cells of the four strains matched their specific catalase activity, in parallel with bacterial growth. In the past decade, the regulation of the catalase levels in a number of other microorganisms has been studied. It appeared that the two main factors influencing the catalas levels in the majority of bacteria are H_2O_2 and the stationary phase response (Loewen, 1997). Oxygen levels have been indicated as a third general factor regulating catalase synthesis in some organisms, like *Bacteroides fragilis* (Rocha and Smith, 1995), *Xanthomonas orzae* (Loprasert *et al.*, 1996) and *Lactobacillus sakei* (Hertel *et al.*, 1998). In addition, there are some regulatory mechanisms that appear to be unique to individual organisms. These include carbon limitation repression (Rocha and Smith, 1997), superoxide induction (Loprasert *et al.*, 1996), repression in stationary phase (Bishai *et al.*, 1994) and cell density repression (Ma and Eaton, 1992; Ansley *et al.*, 1995).

The catalase expression in the four strains of *B. cepacia* used in this study increased when planktonic cells entered the stationary phase. However, activity was not totally depressed at mid-log phase, in highly active strains like the J2315 and JL27. This might indicate that the catalase is constitutively expressed in these strains.

The catalase expression of *B. cepacia* J2315, J2552, JL27 and JL32 may raise the question of whether catalase from these strains is under the control of the stationary phase sigma factor RpoS. RpoS homologues, also known as KatF, have been found in a number of bacteria, but most work on the RpoS regulon has been carried out in *Escherichia coli*. RpoS plays a key role in the survival of bacteria during starvation or exposure to stress conditions and is required for the expression of many genes in stationary phase (Ishihama, 1997; Loewen *et al.*, 1998; Hengge-Aronis, 1999). During rapid growth in laboratory conditions, *E. coli* contains very little RpoS, which becomes abundant at the onset of the stationary phase. However, it was shown that RpoS expression was also induced during exponential (Log) phase upon exposure to certain stress conditions (Ishihama, 1997; Loewen *et al.*, 1998; Hengge-Aronis, 1999). The cellular level of RpoS is regulated at the level of transcription, translation and protein stability. Currently it is predicted that at least 50 genes are subject to RpoS regulation in *E. coli* (Loewen, 1998; Tao *et al.*, 1999) amongst which is the *katE* gene. Induction of *katE* in sationary phase suggested a role in long term survival under starvation and stress conditions. As the catalase expression from highly active strains of *B. cepacia* used in this study, especially J2315, were observed at mid-log as well as early stationary phase, this might indicate that the catalase gene is not regulated by levels of RpoS at different growth stages.

In comparison to planktonic cells, biofilm cells of *B. cepacia* strains J2315, J2257, JL27 and JL32, showed reduced catalase activity in mid-log phase, and further reduction was observed on entering stationary phase. Also the susceptibility of strains to H_2O_2 was increased in biofilm cultures when compared with planktonic ones.

These data indicated that biofilm growth was indeed affecting the oxidative response in *B. cepacia* strains. One likely possibility is the direct effect of environmental factors, on gene regulation, that may cause the different response to oxidative stress response in *B. cepacia* strains. The most obvious environmental factors that could affect catalase expression in biofilms and potentially account for this difference are iron, oxygen availability and H_2O_2 production. Oxygen availability is a possible explanation for the depressed levels of catalase in biofilm cutures of *B. cepacia* strains. It is worth noting that catalase activity and resistance of biofilm cells, were higher at mid-log phase where the number of cells is less and oxygen availability is greater than at stationary phase. Other studies have shown that oxygen tension is reduced in bacterial biofilms (Costerton *et al.*, 1995; Xu *et al.*, 1998) and the endogenous generation of H_2O_2 also will be reduced and consequently the requirement for elevated catalase levels will be minimal.

This result is consistent with the previous studies by Hassett *et al.*, (1999a and b.) who observed that catalase activity (KatA) of *P. aeruginosa* biofilm cells was considerably lower than that in planktonic cells, but the resistance of biofilm *P. aeruginosa* to H_2O_2 was greater than their planktonic counterparts. Frederick *et al.*, (2001) observed that depressed catalase activity from biofilm cells of *P. auroginosa* was due to iron limitation and there was no evidence of influence oxygen limitation. In a recent study,

Wood and Sørensen (2001) showed that catalase levels from biofilm cells of *Nitrosomonas europaea* were elevated over 50% when compared with planktonic cells. The increase in catalase activity was attributed to the response of cell density-dependent gene, which expression resulted in the accumulation of homoserine lactone (HSL) within the constrained intercellular spaces of biofilms. However, Wood and Sørensen showed no evidence for a role of HSL-mediated gene regulation of catalase from *Nitrosomonas europaea* (Wood and Sørensen. 2001).

Previous studies showed evidence that bacteria in the lung grow as a biofilm and under iron-limited (Brown *et al.*, 1984; Haas *et al.*, 1991), oxygen-starved conditions (Costerton *et al.*, 1995; Xu *et al.*, 1998) and in immediate vicinity to increased concentrations of H_2O_2 produced from stimulated neutrophils. In this study, biofilm cells of *B. cepacia* strains were not exposed to iron starvation and their catalase activities were low as well as their resistance to oxidative stress were attenuated. Accordingly, resistance of *B. cepacia* strains to oxidative stress cannot, at this point, be completely attributed to differences in catalase activity. An alternative explanation might be that cells grown in liquid are exposed to a different oxidizing environment from those growing as a biofilm, resulting in an altered expression of stress enzymes or there could be direct effects of environmental factors on the enzymes themselves. Another possibility is that catalase genes from *B. cepacia* may be modulated by other regulatory genes not yet identified.

The ability of *B. cepacia* strain to persist and survive intracellularly despite the oxidative stress in the CF lung may be attributed in part to the antioxidant defence mediated by catalase, while the antioxidant enzyme MsrA likely plays no role in the

oxidative response. In addition, there was some variation of catalase activity among strains of *B. cepacia,* which may explain, in part, the variation in virulence among strains of *B. cepacia*. Other studies detected differences in the pathogenic stimulatory activities of the lipopolysaccharide (LPS) from *B. cepacia*, *P. aeruginosa* and *Stenotrophomonas maltophilia* which could explain the different pathogenic sequelae observed with lung infections caused by these three organisms in CF patients (Shaw *et al.*, 1995; Demko *et al.*, 1998; Zughaier *et al.*, 1999).

In summary, the resistance of *B. cepacia* strains to toxicity by reactive oxygen species may be explained, at least in part, by their ability to produce catalase antioxidant enzyme. The level of expression of catalase for the most virulent strain, J2315, was detected at mid log and early stationary and its specific activity increased in the cells entering stationary phase. This may be important *in vivo* as it would provide a higher level of protection to this strain able to establish a chronic infection where the bacteria would likely be under physiological conditions similar to the late stationary phase of growth. Alternatively, altered resistance of *B. cepacia* strains growing in biofilm mode, resembling those colonizing the airways, to oxidative killing, suggested that there might be another virulence factor(s) contributing to their resistance and ability to induce inflammation in the CF lung.

Cloning and analysis of a deduced sequence of the structural gene encoding HPII catalase could be used to isolate and sequence the regulatory genes upstream, or could be used as a probe to identify variations in expression of catalase product from other

strains of *B. cepacia*. The catalase gene from J2315 and J2552 were selected to be isolated because their catalase activity, as well as their resistance to H_2O_2, were different. The first step to isolate catalase from *B. cepacia* J2315 and J2552 was to find sequences of homology after pair alignment of *E. coli* and *P. auroginosa*. As the 550 bp segments, we predicted to be part of the catalase gene, were successfully isolated and cloned from the two selected strains, only the deduced segment from J2315 were sequenced. The HPII deduced segment from the nucleotide sequence of *katE* of J2315 showed a great degree of similarity with other catalase from related bacteria. Several of the conserved amino acids in *B. cepacia* J2315 catalase correspond to residues of amino acids in that play a role in active site of the *E.coli* HPII and in binding the protoheme. In particular, one of the three amino acid residues highly conserved in the active site of the enzyme of *E.coli* HPII and had its counterpart in *B. cepacia* J2315, was Asn-201. Another one residues of the seven highly conserved residues on the proximal side of the heme in the of *E. coli* HPII sequence Phe-206 was also conserved on the sequence of the *B. cepacia* J2315 catalase sequence. Finally, one of the four conserved amino acids involved in NADPH binding in the HPII sequence of *E. coli*, were also found in the catalase segment of *B. cepacia* J2315 strain which is the Lys-294 (vonOssowski *et al.*, 1991). From these data, we can conclude that the cloned segment contains the gene coding the catalase HPII catalase of *B. cepacia* J2315. The cloning and the expression of *B. cepacia katE* gene could enable biochemical characterization of protein structure and function.

5. General Discussion

Understanding host-pathogen interactions and selective pressures produced by inflammation of the respiratory tract in the CF airway is an extensive goal of cell biology to monitor the leading cause of morbidity and mortality in CF. The characteristic feature of the CF lung disease is the inability of the host to clear the initial colonization and infection of the airways despite an intense inflammatory response. An influx of neutrophils and macrophages and the secretion of pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α characterize this inflammatory process. Proteases, oxidants and α-defensins are released from inflammatory cells and contribute further to tissue destruction. DNA from lysed neutrophils, epithelial cells and bacteria increase the viscosity of airway secretions, leading to further impairment of mucociliary function. Exhaled air from patients with CF contains less nitric oxide (NO) than that from normal controls, indicating increased metabolism of NO or decreased production due to down regulation of inducible nitric oxide synthase (Kelley and Drumm, 1988; Grasemann *et al.*, 1997). The respiratory epithelial cells are a major source of iNOS and NO in the lung where iNOS is expressed constitutively. The deficiency of NO in CF epithelia may contribute, in part, to the susceptibility of these patients to bacterial colonization. Accordingly, the question arises: **How and why is iNOS expression down regulated in CF respiratory epithelium?** .

In vitro as well as *in vivo* studies suggested that CFTR may be involved in maintaining the antioxidant homeostasis of the pulmonary epithelial lining fluid, and

a mutation in CFTR could make the CF lung more susceptible to oxidative stress (Gao *et al.*, 1999; Velsor *et al.*, 2001). The oxidative environment is created by the inflammatory response and from the infectious agents. In many systems, the presence of oxidative stress increases cytokine-mediated iNOS expression (Kuo *et al.*, 1997), and more interestingly, other studies demonstrated that reactive oxygen intermediates induce activation of NF-κB, a transcriptional factor essential for the expression of iNOS (Hoare *et al.*, 1999). However, oxidants, including H_2O_2, are known to activate PI 3-kinase pathway (Shaw *et al.*, 1998; Sonoda *et al.*, 1999). In many studies it was demonstrated that inhibition of PI 3-kinase is necessary for the expression of iNOS (Wright *et al.*, 1997; Park *et al.*, 1997; Pahan *et al.*, 1999). It is conceivable that in CF, high oxidative stress may activate PI 3-kinase, which in turn downregulates iNOS expression. This hypothesis was justified by activating PI 3-kinase from human A549 respiratory epithelial cell line via IL-13, which reduced NO production. Cytokine-stimulated A549 cells expressed elevated levels of NO compared with unstimulated cells that expressed minimal constitutive NO. There are a number of studies that support the view that PI 3-kinase activity down-regulates iNOS and that suppressors of iNOS need to activate PI 3-kinase in order to inhibit iNOS transcription and /or activity. Interleukin-13 has been shown to inhibit iNOS expression from A549 cell line (Berkman *et al.*, 1996) but this work is the first to provide evidence for the involvement of the activity of PI 3-kinase. The use of PI 3-kinase inhibitors, wortmannin and LY249002, restored NO levels in cytokine-stimulated A549 pretreated with IL-13. This indicated that IL-13 inhibited NO in A549 indirectly by its ability to activate PI 3-kinase in A549 cells. However, other

studies demonstrated an alternative way by which IL-13 can inhibit NO expression. IL-13 was shown to up-regulate arginase, another enzyme that utilizes the iNOS substrate L-arginine, and thus reduces NO production (Chang *et al.*, 2000; Rutschman *et al.*, 2001). However, arginase activity was not detected from cytokine stimulated A549 cells pretreated with IL-13. This might eliminate the possibility for IL-13-inhibited NO production via arginase. Accordingly, inhibition of NO from cytokine stimulated A549 inhibition pre-treated with IL-13 was through the activation of PI 3-kinase *per se.*

These data show evidence which implicate PI 3-kinase-dependent signaling in the regulation of iNOS activity in CF human respiratory epithelial cells. However, it is worth emphasising that experiments investigating the regulation of NOS by PI 3-kinase have used many different cell models, and accordingly the interaction of PI 3-kinase with iNOS may exhibit tissue-specific regulation.

If iNOS expression in bronchial epithelium of CF patients was shown to be deficient, what difference does it make? Nitric oxide, expressed in higher concentration from activated iNOS, is a simple molecule that has been implicated in a vast array of physiological functions, including vasoregulation, neurotransmission and immune regulation. Nitric oxide and its oxidative by products, RNS, inhibit microbes in a manner that is similar to the mechanisms by which RNS inhibit mammalian target cells (Nathan and Hibbs, 1991). Most importantly, during an inflammatory response NO, or RNS, can be produced concurrently with superoxide anions, or ROS, in nearly equimolar amounts and can generate the particularly destructive product, peroxynitrite ($OONO^-$) (Zhu *et al.*, 1992). Smith *et al.*, (1999)

demonstrated the synergistic effect of NO with reactive oxygen species to eradicate one of the most important pulmonary pathogen in CF *B. cepacia in vitro*. So, deficiency of NO may contribute partially to enhanced susceptibility of CF patients to respiratory tract infections. So, since CF disease involves organs other than the respiratory tract, **why is respiratory infection the major concern?** In patients with cystic fibrosis, respiratory infection is the major cause of morbidity and mortality. With the development of treatments for intestinal obstruction and pancreatic insufficiency, patients typically survive beyond infancy, and at some point, virtually all patients develop chronic bacterial infection, abnormal airway secretions and airway inflammation (Pilewski and Frizzeill, 1999). This explains why lots of researchers aim to study the interaction between infection, inflammation and pulmonary function in CF patients. Common bacterial pathogens that infect the CF lung include *Staphylococcus aureus*, *Haemophilus influenzae*, *P. aeruginosa* and *B. cepacia*.

Infection with such opportunistic bacteria indicates the milieu of the lung of CF patients provides a favorable niche for bacterial infection. Among all, the most important pathogen which has emerged recently is infection with *B. cepacia*. So, **why is infection with *B. cepacia* problematic in CF patients?** *Burkholderia cepacia* is an extremely diverse class of bacteria, and the taxonomy has evolved to accommodate the enhanced understanding of the new genus within which it has been placed (Yabuuchi *et al.*, 1992). Infection with *B. cepacia* strains, typically acquired late in the lifetime of CF patients, is most of the time preceded by infection with *P. aeruginosa* (Riedel *et al.*, 2001). Apart from acquisition from the natural habitat,

patient-to patient transmission and indirect nosocomial acquisition from contaminated surfaces have caused several outbreaks within and between regional CF centres (Govan and Deretic, 1996). Among the key strategies of *B. cepacia* to persist in the CF lungs resistance to phagocytic killing and invasion of epithelial cells, intrinsic resistance to a broad range of antibiotics (Tummler and Kiewitz, 1999) as well as its resistance to epithelial-derived antimicrobial substances (Baird *et al.*, 1999) are likely important. Moreover, differential pathogenicity among strains within the complex has been observed in CF patients. Colonization with strains of *B. cepacia* may range from chronic asymptomatic carriage to a rapidly fatal decline in lung function. Approximately, 80% of *B. cepacia* clinical isolates from Canada are from genomovar III, the most highly transmissible strains also belong to this genomovar, which is also associated with a higher mortality rate than other members of the complex (Speert *et al.*, 2002). So, **why do different genomovars of *B. cepacia* complex strains elicit different disease progression?** Genomovars are phenotypically similar but genotypically distinct groups of strains that show low level of DNA hybridization. It was recently demonstrated that differential persistence occurred among genomovars of *B. cepacia* complex in a murine model of pulmonary infection (Chu *et al.*, 2002), however, details of which virulence factors were related to these differences were not described. Strains of *B. cepcia* complex produce a number of potential virulence factors, as mentioned in **Introduction**, and three markers have been predominantly found *B. cepacia* genomovar III associated with its transmissible isolates, including cable pili (Sun *et al.*, 1995), which have been shown to mediate adherence to respiratory mucin (Sajjan *et al.*, 1995); an open reading

frame with homology to transcriptional regulators termed the *B. cepacia* epidemic strain marker, but its function is not well characterised (Mahenthiralingam *et al.*, 1997); and a hybrid of insertion sequences IS*402* and IS*1356* (Tyler *et al*, 1996). Yet, up till this time there is no evidence to suggest that some strains are more virulent than others. **What about the setting of the CF lung?** Many reports described the relationship between the defect in CFTR function and the increased susceptibility of CF lung to infection (Pilewski and Frizzell, 1999). The unique setting of the CF lung and airways may contribute to the possible escape of *B. cepacia* from oxidative killing. Moreover, it was shown by Isles *et al.* (1984) that differences in clinical features of pulmonary infection with strains of *B. cepacia* are related to differences in the underlying clinical conditions of the lung prior to infection or alternatively to infection by different strains. As mentioned earlier, recruitment of neutrophils to the site of infection is associated with a release of reactive oxygen species. In a recent study by Lefebre and Valvano (2001), seven out of 11 strains displayed high-level survival after H_2O_2 treatment *in vitro* which had a bifunctional catalase/peroxidase. These isolates, including genomovar III strains, showed different levels of resistance to intracellularly generated O_2^- and were correlated with detected levels of SOD activity. However, differences in survival to extracellular O_2^- were not detected. Furthermore, a recent study showed that nitric oxide acts synergistically with reactive oxygen species to kill *B. cepacia in vitro* (Smith *et al.*, 1999) and suggested that persistence of *B. cepacia* in CF patients may be attributed to the defect in the iNOS activity in these patients (Kelley and Drumm, 1998). In another study, the presence of *B. cepacia* infection involved in CGD patients, whose neutrophils do not

express an oxidative respiratory burst (Speert *et al.*, 1994), supports the proposal that reactive oxygen and nitrogen species both can eradicate the *B. cepacia* infection. Also Smith *et al.*, (1999) demonstrated that catalase from a number of tested *B. cepacia* strains and not SOD was able to abolish peroxynitrite killing. Then the attractive hypothesis that *B. cepacia* strains may vary in their catalase expression stemmed from a precedent observation of Smith's study in which preliminary data showed variations in resistance of different strains of *B. cepacia* to killing by H_2O_2, and when synergies with NO. The question then comes, **Are there differences in expressed catalases from *B. cepacia strains*?** First of all it was necessary to establish the difference in the catalase expression and resistance to H_2O_2. In this study, four different strains from *B. cepacia* complex were investigated, differences in catalase specific activities where determined and the *B. cepacia* JL27 and J2315 strains, belonging to genomovar III was found with highest catalase activity, and highest resistance to oxidative killing with H_2O_2 compared to the other strains J2552 and JL32. To understand better catalase expression, and resistance of *B cepacia* strains to killing by H_2O_2, *B. cepacia* were grown on a membrane surface resembling biofilm culture. It is now widely accepted that infections of soft tissues, bacteria grow as biofilms and such a clinical setting is very important, especially in CF patients (Gilbert and Brown, 1995). Biofilms may be regarded as adherent microcolonies, forming functional association of cells embedded within an exopolysaccharide. Growth in this manner is clearly of advantage to bacterial cells living within a harsh environment since bacterial cells can persist in spite of antimicrobial therapy (Brown and Williams, 1985) and immune response (Anwar *et*

al., 1983). Most importantly, genetic studies demonstrated variations in gene transcription in planktonic and biofilm-associated phases of the bacterial life cycle (Watnick and Kolter, 2000). In Gram-negative microbial biofilm gene expression of certain virulence factors is in a cell-density dependent manner. This form of gene regulation is widely known as quorum sensing (QS). Thus, during biofilm growth bacteria may express many stationary phase specific genes, of such is the monofunctional catalase gene. We thought that biofilm *B. cepacia* might express catalase in higher concentrations resembling planktonic cells at stationary phase. Unfortunately, the catalase activity and resistance of biofilm *B. cepacia* strains were reduced and became more susceptible to H_2O_2 compared with planktonic cells. However, while catalase was implicated as an important factor in resistance to H_2O_2 lethal affect, other factors seemed critical in controlling catalase expression from *B. cepacia* strains in biofilms.

As there is no doubt the contribution of catalase to protect strains of *B. cepacia* from damage caused by reactive oxygen species in CF lung, other environmental factors that may affect regulation of expression of catalase should be taken into consideration. Such environmental factors like iron availability and environmental acidity (Olson, 1993; Foster, 1999) if vary during infection may be in the favour of the pathogen.

Are there any other virulence factors that may contribute to the resistance of *B. cepacia* phagocytic oxidative killing? The mechanisms by which intracellular pathogens resist killing by macrophages include inhibition of phagosome-lysosome fusion, escape into the cytoplasmic compartment and resistance to reactive oxygen

intermediates and lysosomal enzymes (Kaufmann, 1993). The intracellular *B. cepacia* cells were observed in infected macrophages as well as in respiratory epithelial cells, enclosed in membrane vacuoles (Burns *et al.*, 1996). This indicates that the *B. cepacia* does not need to escape into cytoplasmic compartment to survival since it can inhibit the antimicrobial response of macrophages. In addition to SOD and catalase, *B. cepacia* strains produce melanin-like pigment, which may act as a scavenger of superoxide radicals (Zughaier *et al.*, 1999). In addition it was observed that engulfment of *B. cepacia* by alveolar macrophages was reduced in the presence of lipase produced by *B. cepacia* cells (Straus *et al.*, 1992). Recently, it was shown that *B. cepacia* produced elevated levels of lipase under oxygen-depleted conditions, which may help cells to evade phagocytic phagocytosis during the host responce (McKenney and Allison, 1995).

6. Conclusion and future work

In this project, it has been shown for the first time that modulation of the cytokine activated iNOS enzyme in pulmonary epithelial cells by the signaling enzyme PI 3-kinase. In addition, the anti-inflammatory cytokine IL-13 can activate this signaling enzyme to down-regulate cytokine stimulated iNOS activity. It was demonstrated that modulation of cytokine-stimulated iNOS from respiratory epithelial cells was not through the activation of arginase, which may compete with iNOS for the common substrate L-arginine.

An important arena for the ability of the PI 3-kinase to mediate iNOS activity is the CF pulmonary epithelium. Increased susceptibility of CF patients was contributed to the absence of iNOS expression from respiratory tract, thus deregulation of the PI 3-kinase or using a selective PI 3-kinase inhibitor in CF may restore iNOS activity. By activating iNOS in the airway epithelium, levels of NO may be restored to potentiate reactive oxygen intermediates to terminate respiratory infectious bacteria is the main cause of lung dysfunction and high mortality and morbidity rate among CF patients.

In view of the PI 3-kinase results, there is much to be done to convert these initial findings in simple system into a more cogent understanding of the role of signaling and iNOS expression in CF physiology and disease. This includes:

- Assay for the PI 3-kinase accumulation in cytokine-stimulated A549 after treatment with IL-13, to further support that IL-13 modulation to cytokine stimulated iNOS is via PI 3-kinase.

- Detection of the ability of IL-13 to modulate NF-κB and activate STAT6 in A549 respiratory cell line.
- Assay for iNOS activity from primary respiratory cell line transfected with CF gene, after cytokine stimulation and IL-13 treatment. This may explain if PI 3-kinase could modulate iNOS expression in CF lungs.

- Develop an oxidative stress system in a respiratory epithelial cell line to detect PI 3-kinase activity as well as activation of NF-κB, which are crucial event in the modulation of iNOS expression.

Another axis to combat respiratory infections in CF patients is to identify and characterize virulent factors that may attribute to the pathogenecity of the most troublesome infecting bacteria *B. cepacia*. The catalase activity, which enables strains to resist oxidative killing during infection, was found variable among 4 different strains of *B. cepacia*. Differences of catalase activity were consistent with the strains' susceptibility to oxidative killing. This may explain difference between strains of *B. cepacia* to oxidative killing. In addition, variations in catalase activity and resistance to oxidative killing between genomovars may also explain reason behind high virulence and transmissibility in CF with genomovar III but rare infectivity with genomovar I (Revets *et al.*, 1996). The results from this study demonstrated that the catalase activity from *B. cepacia* strains when growing as biofilm, the way infectious bacteria grow in CF, were attenuated. Hence, the results of catalase activity open the gate for further aspects to be investigated:

- Assay for catalase activity of planktonic and biofilm of *B. cepacia* when grown under iron limitation. This may indicate if iron which is essential, for catalase synthesis plays a role in catalase expression in *B. cepacia* strains.
- Since catalase was detected at mid-log as well as early stationary phase it may be quite novel to investigate if induction of monofunctional catalase is under the regulation of the RpoS regulon.
- It may be quite feasible if the A549 cell line could be used to test survival of *B. cepacia* when stimulated with pro-inflammatory cytokines in the presence and absence of IL-13 treatment.

In a first step towards investigating catalase expression at molecular level a deduced sequence of the structural catalase gene from *B. cepacia* J2315, that belongs to the most pathogenic group genomovar III, have been isolated, cloned and sequenced.

Understanding factors that contribute to the regulation of oxidative stress response should also aid in the dissection of host-pathogen interactions associated with CF respiratory infections. The catalase segment of the J2315, may be used in the future work to:

- Isolate and sequence regulatory genes upstream that are involved in the regulation of catalase expression. Investigate further, factor(s) that may induce the expression of these regulon(s) and eventually control catalase expression.

- Study catalase gene expression related to *B. cepacia* biofilms to understand if the regulation of these genes under stress conditions.
- Sequnence the isolated and cloned 550bp deduced segment of *B. cepacia* J2552 strain and screen the conserved amino acid residues and its homology to the sequenced *B. cepacia* J2315 strain. Since both strains exhibited different catalase activities and resistance to oxidative killing.

In conclusion, the data presented in this study have identified methods that can modulate synthesis of reactive nitrogen species from activated inducible nitric oxide synthase of pulmonary epithelial cells. In addition it demonstrated that strains of *B. cepacia* that are CF bacterial pathogens, have variable susceptibility to hydrogen peroxide. Furthermore, such findings contribute to a more thorough understanding of the host responses of the respiratory tract, and how they are disturbed in CF. It may allow a better understanding for methods to manipulate reactive nitrogen and oxygen species production and reactivity to improve the immunity in CF respiratory which may prevent or alleviate the fatal pulmonary disease in such patients.

7. REFERENCES

Alderton, W.K., Cooper, C. E. and Knowles, R.G. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem. J.*, **357**: 593-615

Ansley, J.C., Georgina, A.D. and Huw, D.W. (1995) Evidence for cell-density dependent regulation of catalase activity in *Rhizobium Leguminosium bv. Phaseoli. Microbiology*, **141**: 843-851.

Anwar, H., Browm, M.R.W. and Lambert, P.A. (1983) Effect of nutrient depletion on sensitivity of Pseudomonas cepacia to phagocytosis and serum bactericidal activity at different temperatures. *J. Gen. Microbiol.*, **129**: 2021-2027.

Armstrong, D., Grimwood, K., Carlin, J.P., Carzino, R., Gutierrez, J.P., Hull, J., Olinsky, A., Phelan, E.M., Robertson, C.F. and Phelan, P.D. (1997) Lower airway inflammation in infants and young children with cystic fibrosis. *Am. J. Respir. Crit Care Med.,* **156**: 1197-1997.

Asano, K., Chee, C.B., Gaston, B., Lilly, C.M., Gerard, C., Drazen, J.M. and Stamler, J.S. (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. USA.*, **91**: 10089-10093.

Baird, R.M., Brown, H., Smith, A.W. and Watson, M.L. (1999) *Burkholderia cepacia* is resistant to the antimicrobial activity of airway epithelial cells. *Immunopharmacology,* **44**: 267-272.

Bals, R., Weiner, D. and Wilson, M. (1999) The innate immune system in cystic fibrosis lung disease. *J. Clin. Invest.*, **103**: 303-307.

Barnes, P.J. and Karin, M. (1997) Nuclear factor -κB- A pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.*, **335**: 1066-1071.

Belvisi, M.G., Ward, J.K., Mitchell, J.A. and Barnes, P.J. (1995) Nitric oxide as a neurotransmitter in human airways. *Arch. Int. Pharmacodyn. Ther.*, **329**: 97-110.

Berkman, N., Robichaud, A., Robbins, R.A., Roesems, G., Haddad, E.B., Barnes, P.J. and Chung, K.F. (1996) Inhibition of inducible nitric oxide synthase expression by interleukin-4 and interleukin-13 in human lung epithelial cells. *Immunology,* **89**: 363-367.

Beutler, B. (1995) TNF immunity and inflammatory disease: lessons of the past decade. *J. Investig. Med.,* **43**: 227-235.

Bishai, W.R., Smith, H.O. and Barcak, G.J. (1994) A peroxide/ascorbate-inducible catalase from *Haemophilus influenzae* is homologous to the *Escherichia coli katE* gene product. *J. Bacteriol.*, **176**: 2914-2921.

Blackwell, T. S., Stecenko, A. A. and Christman, J. W. (2001) Dysregulated NF-κB activation in cystic fibrosis: evidence for a primary inflammatory disorder. *Am. J. Physiol. Lung cell Mol. Physiol.,* **281**: L69-L70.

Blantz, R.C., Striano, F., Gabbai, F. and Kelly, C. (2000) Biological effects of arginine metabolites. *Acta Physiol Scand.,* **168**: 21-25.

Boehm, U., Klamp, T., Groot, M. and Howard, J.C. (1997) Cellular responses to interferon-gamma. *Immunology,* 15: 749-795.

Bonfield, T.L., Konstan, M.W., Burfeind, P., Panuska, J.R., Hilliard, J.B. and Berger, M. (1995) Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is down regulated in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.*, **13**: 257-261.

Bonfield, T.L., Panuska, J.R., Konstan, M.W., Hilliard, K.A., Hilliard, J.B., Ghniam, G.B. and Berger, M. (1995) Inflammatory cytokines in cystic fibrosis lungs. *Am. J. Respir. Crit. Care Med.*, **152**: 2111-2118.

Bollinger, N., Hassett, D.J., Iglewski, B.H., Costerton, J.W. and McDermott, T.R. (2001) Gene expression in *Pseudomonas aeruginosa*: evidance of iron override effects on quorum sensing and biofilm-spesific gene regulation. *J. Bacteriol.*, **183**: 1990-1996.

Boucher, R.C., Stutts, M.J., Knowles, M.R., Cantley, L. and Gatzy, J.T. (1986) Na^+ Transport in cystic fibrosis. Abnormal basal rate and response to adenylate cyclase activation. *Am. Soci. Clin. Invest.*, **78**: 1245-1252.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248-254.

Bradley, L., Dalton, D.K. and Croft, M. (1996) A direct role of IFN- γ in regulation of Th1 cell development. *J. Immunol.*, **157**: 1350-1358.

Brombacher, F. (2000) The role of interleukin-13 in infection disease and allergy. *Bioessays*, **22**: 646-656.

Brot, N.L., Weissbach, J.W. and Weissbach, H. (1981) Enzymatic reduction of protein-bound methionine sulfoxide. *Proc. Natl. Acad. Sci. USA.*, **78**: 2155-2158.

Brot, N.L., Werth, J., Koster, D. and Weissbach, H. (1982) Reduction of N-acetyl methionine sulfoxide: a simple assay for peptide methionine sulfoxide reductase. *Anal. Biochem.*, **122**: 291-294.

Brown, M.R.W., Anwer, H. and Lambert, P.A. (1984) Evidence that mucoid Pseudomonas aeruginosa in the cystic fibrosis lung grows under iron–restricted conditions. *FEMS Microbiol. Lett.*, **21**: 113-117.

Brown, M.R.W. and Williams, P. (1985) Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. *J. antimicrob. Chemother.*, **15**: 7-14.

Brown, S.M., Howell, M.L., Vasil, M.L., Anderson, A.J. and Hassett, D.J. (1995) Cloning and characterization of the *katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peoxide- inducible catalase: purification of KatB, cellular localization and demonstration that it is essential for optimal resistance to hydrogen peroxide. *J. Bacteriol.*, **177**: 115-117.

Brunelli, L., Crow, J. P. and Bakman, J. S. (1995) The comparative toxicity of nitric oxide and peroxynitrite to *Escherishia coli. Arch. Biochem. Biophys.*, **316**: 327-334.

Bryk. R., Griffin, P. and Nathan, C. (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature*, **407**: 211-215.

Buga, G. M., Wei, L. H. and Bauer, P. M. (1998) N^G-hydroxyl-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanism. *Am. J. Physiol.*, **44**: R1256-R1264.

Buhler, T., Ballestero, S., Desai, M. and Brown, M.R.W. (1998) Generation of reproducible nutrient-depleted biofilm of *Escherichia coli* and *Burkholderia cepacia*. *J. Appl. Microbiol.*, **85**: 457-462.

Burns, J.L., Clark, D.K. and Wadsworth, C.D. (1992) Proc. 6th. *Annu. N. Am. Cystic Fibrosis Conf., abstr.* **201**.

Burns, J.L., Jonas, M. and Chi, E. (1996) Invasion of respiratory epithelium by *Burholderia* (*Pseudomonas*) *cepacia. Infect. Immun.*, **64**: 4054-4059.

Cairns, G., Kerr, K.G., Beggs, C.B., Sleigh, P.A., Mooney, L., Keig, P. and Donnelly, J.K. (2001) Susceptibility of *Burkholderia cepacia* and other pathogens of importance in cystic fibrosis to U.V. light. *Lett. Appl. Microbiol.*, **32**: 135-138.

Cantin, A. (1995) Cystic fibrosis lung inflammation: early, sustained and severe. *Am. J. Respir. Crit Care Med.*, **151**: 939-941.

Cantrell, D.A. (2002) Phosphoinositide 3-kinase signaling pathways. *J. Cell Sci.*, **114**: 1439-1445.

Casado, M., Diaz-Guerra, M., Bosca, L. and Martin-Sanz, P. (1997) Differential regulation of nitric oxide synthase mRNA expression by lipopolysaccharide and pro-inflammatory cytokines in fetal hepatocytes treated with cycloheximide. *Biochem. J.*, **327**: 819-823.

Chang, C.I., Lio, J.C. and Kuo, L. (1998) Arginase modulates nitric oxide production in activated macrophages. *Am. J. Physiol.*, **247**: H342-348.

Chang, C.I., Zoghi, B., Liao, J.C. and Kuo, L. (2000) The involvement of tyrosine kinases, Cyclic AMP/protein kinaseA and p38 mitogen-activated protein kinase in IL-13-mediated arginase I induction in macrophages: its implications in IL-13 inhibited nitric oxide production. *J. Immunol.*, **165**: 2134-2141.

Cheng, S., H., Rich, D.P., Arshall, J., Gregory, R.J., Welsh M.J. and Smith, A. E. (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell*, **66**: 1027-1036.

Chiu, C., Wong, S., Hancock, W. and Spreet, D. (2001) Adherence *Burkholderia cepacia* to respiratory tract epithelial cells and inhibition with dextran. *Microbiology,* **147**: 2651-2658.

Chmiel, J.F., Konstan, M.W., Knesebeck, J.E., Hillard, J.B., Bonfield, T.L., Dawson, D.V. and Berger, M. (1999) IL-10 attenuates excessive inflammation in chronic Pseudomonas infection in mice. *Am. J. Respir. Crit. Care. Med.*, **160**: 2040-2047.

Christman, M.F., Morgan, R.W., Jacobson, F.S. and Ames, B.N. (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium. Cell,* **41**: 753-762.

Chu, S.C., Marks-Konczalik, J., Wu, H., Banks, T.C. and Moss, J. (1998) Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: Characterization of difference between human and mouse iNOS promoters. *Biochem Biophys. Res. Commun.*, **248**: 871-878.

Chu, K.K., Davidson, D.J., Halsely, T.K., Chung, J.W. and Speert, D.P. (2002) Differential persistence among genomovars of the *Burkholderia cepacia* complex in a murine model of pulmonary infection. *Inf. Immunol.*, **70**: 2715-2720.

Chung, C., Liao, J.C. and Kuo, L. (1998) Arginase modulates nitric oxide production in activated macrophages. *Am. J. Physiol.*, **274**: H342-H348.

Clare, D.A., Duong, M.N., Darr, D., Archibald, F.and Fridovich, I (1984) Effects of molecular oxygen on the detection of superoxide radical with nitroblue tetrazolium and activity stain for catalase. *Anal. Biochem.*, **140**: 532-537.

Collins, F.S. (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science,* **256**: 774-779.

Comhair, S.A.A. and Erzurum, S.C. (2002) Antioxidant response to oxidant – mediated disease. *Am. J. Physiol. Lung Mol. Physiol.*, **283**:L246-L255.

Conneely, L., Palacios-Callender, M., Ameixa, C., Moncada, S. and Hobbs, A. J. (2001) Biphasic regulation of NF-κB activity underlies the pro- and anti-inflammatory action of nitric oxide. *J. Immunol.*, **166**: 3878-3881.

Cooper, P., Potter, S., Mueck, B., Yousefi, S. and Jari, G. (2001) Identification of genes induced by inflammatory cytokines in airway epithelium. *Am. J. Physiol. Lung Mol. Physiol.*, **280**: L841-L852.

Corraliza, I.M., Campo, M.L., Soler, G. and Modollel, M. (1994) Determination of arginase activity in macrophages: a micromethod. *J. Immunol. Methods,* **174**: 231-235.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korbe, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu. Rev. Microbiol.*, **49**: 711-745.

Dakin, C.J., Numa, A.H., Morton, J.R., Vertzyas, C.C. and Henry, R.L. (2002) Inflammation, infection and pulmonary function in infants and young children with cystic fibrosis. *Am. J. Respir. Crit. Care Med.*, **165**: 904-910.

Darling, P., Chan, M., Cox, A.D. and Sokol, P.A. (1998) Siderophore production by cystic fibrosis isolates of Burkholderia cepacia. *Infect. Immun.,* **66**: 874-877.

Davies, G.D., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costeron, J.W. and Greenberg, E.P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science,* **280**: 295-298.

Davies, J.C., Stern, M., Dewer, M., Caplen, N.J., Munkonge, F.M., Pitt, T., Srgi, F., Huang, L., Bush, A., Geddes, D.M. and Alton, E.W. (1997) CFTR gene transfer reduces the binding of *Pseudomonas aeruginosa* to cystic fibrosis respiratory epithelium. *Am. J. Respir. Cell Mol. Biol.*, **16**: 657-663.

De Assis, M.C., Da Costa, A.O., Barja-Fidalgo, T.C. and Plotkowski, M.C. (2000) Human endothelial cells are activated by interferon –γ plus tumour necrosis factor-α to kill intracellular *Pseudomonas aeruginosa. Immunology*, **101**: 271-278.

De Kievit, T.R. and Iglewiski, B.H. (2000) Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.*, **68**: 4839-4849.

Demko, C.A., Stern, R.C. and Doershuk, C.F. (1998) *Stenotrophomonas maltophilia* in cystic fibrosis: incidence and prevalence. *Pediatr. Pulmonol.*, **25**: 304-308.

Denning, G.M., Wollenweber, L.A., Railsback, M.A., Cox, C.D., Stoll, L.L. and Britigan, B.L. (1998) *Pseudomonas pyocyanin* increases interleukin-8 expression by human airway epithelial cells. *Infect. Immun.,* **66**: 5777-5784.

Deretic, V. Schurr, M.J. and Yu, H. (1995) *Pseudomonas aeruginosa*, mucoidy and chronic infection phenotype in cystic fibrosis. *Trends Microbiol.,* **3**: 351-356.

De Sanctis, G.T., Mehta, S., Kobzik, L., Yandora, C., Jiao, A., Huang, P.L. and Drazen, J.M. (1997) Contribution of type I NOS to expired gas NO and bronchial responsiveness in mice. *Am. J. Physiol.*, **273**: L883-L888.

Diamond, G., Russell, J.P. and Bevnis, C.L. (1996) Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc. Natl. Acad. Sci. USA.*, **93**: 5156-5160.

Diamond, G., Legarda, D. and Ryan, L.K. (2000) The innate immune response of respiratory epithelium. *Immunol. Rev.*, **173**: 27-38.

Diamond, J.P., Diamond, G., Tarver, A.P., Scanlin, T.F. and Bevins, C.L. (1996) Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor-α. *Infect. Immun.*, 64: 1565-1568.

Diaz-Guerra, M.J.M., Castrillo, A., Martin-Sanz, P. and Bosca, L. (1999) Negative regulation by phosphatidylinositol 3-kinase of inducible nitric oxide synthase expression in macrophages. *J. Immunol.*, **162**: 6184-6190.

Doherty, T.M., Kasteelein, R., Menon, S. andrade, S. and Coffman, R.L. (1993) Modulation of murine macrophage function by IL-13. *J. Immunol.*, **151**: 7151-7160 .

Donaldson, A., Daphna-Iken, D., Tetsuka, T. and Morrison, A.R. (1996) Interleukin-1 beta activates PI 3-kinase in renal mesangial cells. *Biochem. Biophys. Res. Commun.*, **227**: 289-293.

Donnelly, L.E. and Barnes, P.J. (2002) Expression and regulation of inducible nitric oxide synthase from human primary airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **26**: 144-151.

Drabick, J.A., Gracely, E.J., Heidecker, G.J. and LiPuma, J.J. (1996) Survival of *Burkholderia cepacia* on environmental surfaces. *J. Host. Infect.,* **32**: 267-176.

Dweik, R.A., Laskowski, D., Abu-Soud, H.M., Kaneko, F., Hutte, R., Stuehr, D.J. and Erzurum, S.C. (1998) Nitric oxide synthesis in the lung. Regulation by oxygen through a kinetic mechanism *J. Clin. Invest.,* **101**: 660-666.

Fan, J., Richard, D. and Malik, A. (2001) Transcriptional mechanisms of acute lung injury. *Am. J. Physiol.*, **281**: L1037-L1050.

Farr, S.B., Nativig, D.O. and Kogoma, T. (1985) Toxicity and mutagenicity of plumbagin and the induction of possible new DNA repair pathway in *Escherichia coli. J. Bacteriol.,* **164**: 1309-1316.

Farr, S.B. and Kogoma, T. (1991) Oxidative stress response in *Escherichia coli* and *Salmonella typhimurium. Microbiol. Rev.,* **55**: 561-585.

Farrant, J.L., Sansone, A., Canvin, J.R., Pallen, M.J., Langford, P.R., Wallis, T.S., Dougan, G. and Knoll, J.S. (1997) Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmolellosis. *Mol. Microbiol.*, **25**: 785-796.

Fang, F.C., Libby, S.J., Buchmeier, N.A., Loewen, P.C, Switala, J., Harwood, J. and Guiney, D.G. (1992) The alternative σ factor KatF (RpoS) regulates *Salmonella virulence. Proc. Natl. Acad. Sci. USA.,* **89**: 11978-11982.

Fang, F. C. (1997) Mechanisms of nitric oxide-related antimicobial activity. *J. Clin. Invest.*, **99**: 2818-2825.

Favre-Bonte, S., Pache, J., Roberst, J., Blanc, D., Pechere, J. and van Delen, C. (2002) Detection of *Pseudomonas aeruginosa* cell-to-cell signals in lung tissue of cystic fibrosis patients. *Microb. Pathog.*, **32**: 143-147.

Fearon, D. and Locksley, R. (1996) The instructive role of innate immunity in the acquired immune response. *Science,* **272**: 50-54.

Ferkol, T.W. and Look, D.C. (2001) Chinks in the armor of the airway, Pseudomonas infection in the cystic fibrosis lung. *Am. J. Respir. Cell Mol. Biol.*, **25**: 11-13.

Fitzgerald, D.M., Cooper, M.P., Tiley, S., Kado, J. and Collins, C. (2001) *Burkholderia cepacia* in cystic fibrosis: Novel Australian cluster strain without accelerated respiratory deterioration. *J. Paediatr. Child. Health*, **37**: 130-136.

FitzSimmons, S.C. (1993) The changing epidemiology of cystic fibrosis. *J. Pediatr.*, **122**: 1-9.

Foster, J.W. (1999) When protons attack: microbial strategies of acid adaptation. *Curr Opin. Microbiol.*, **2**: 170-174.

Freeman, B., Gutierrez, H. and Rubbo, H. (1995) Nitric oxide: a central regulatory species in pulmonary oxidant reactions. *Am. J. Physiol.*, **268**: L697-L699.

Fridovich, I. (1978) The biology of oxygen radicals. Science, **201**: 875-879.

Fruman, D.A., Meyers R.E. and Cantley, L.C. (1998) Phosphoinositide kinase. *Annu. Rev. Biochem.*, **67**: 481-507.

Fruman, D.A. and Cantley, L.C. (2002) Phosphoinositide 3-kinase in immunological systems. *Semin. Immunol.*, 14: 7-18.

Fuqua, C., Winans, S.C. and Greenberg, E.P. (1996) Census and consensus in bacterial ecosystem: the LuxR-Lux1 family of quorum sensing transcriptional regulators. *Annu. Rev. Microbiol.*, **50**: 727-751.

Garvey, E.P., Oplinger, J.A., Furfine, E.S., Kiff, R.J., Laszlo, F., Whittle, B.J. and Knowles, R.G. (1997) 1400W is a slow, tightly binding and highly selective inhibitor of inducible nitric oxide symthase *in vitro* and *in vivo. J. Biol. Chem.*, **272**: 4959-4963.

Gao, L., Kim, K.J., Yankaskas, J.B. and Forman, H.J. (1999) Abnormal glutathione transport in cystic fibrosis airway epithelia. *Am. J. Physiol.*, **277**: L113-L118.

Geisenberger, O., Givskov, M., Riedel, K., Hioby, N., Tummmmler, B. and Eberl, L. (2000) Production of N-acyl-L hormoserine lactones by *Pseudomonas aeruginosa* isolates from chronic lung infections associated with cystic fibrosis. *FEMS Microbiol. Lett.*, **184**: 273-278.

Geller, D.A., Nussler, A.K., DiSilvio, M., Lowenstein, C.J., Shapiro, R.A., Wang, S.C., Simmons, R.L. and Billiar, T.R. (1993) Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. USA.*, **90**: 522-526.

Gilbert, P. and Brown, M.R.W. (1995) Mechanism of the production of bacterial biofilms from antimicrobial agents. In microbial Biofilms ed. Lappin-Scott, H.M. and Costernton, J.W. Cambridge: Cambridge University Press.

Goldman, M.J., Anderson, G.M., Stolzenberg, E.D., Kari, U.P., Zasloff, M. and Wilson, J.M. (1997) Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell*, **95**: 1005-1015.

Gottesman, S. (1984) Bacterial regulation: global regulatory networks. *Annu. Rev. Genet.*, **18**: 415-441.

Govan, J.R., Brown, P.H., Maddison, J., Doherty, C.J., Nelson, J.W., Dodd, M., Greeing, A.P. and Webb, A.K. (1993) Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *Lancet,* **342**:15-19.

Govan, J.R.W. and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia. Microbiol. Rev.,* **60**: 539-574.

Govan, J.R., Hughes, J.E. and Vandamme, P. (1996) *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J. Med. Microbiol.*, **45**: 395-407.

Govan, J.R.W. and Vandamme, P. (1998) Agricultural and medical microbiology-a time for bridging gaps. *Microbiology*, **144**: 2373-2375.

Govan, J.R.W. (2002) An update on the *Burkholderia cepacia* complex. *J. Cystic Fibrosis*, 1: S14.

Granger, D.L., Hibbs, J.B., Perfect, J.R. and Durack, D.T. (1990) Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Inevst.*, **85**: 264-273.

Grasemann, H.E., Michler, E., Wallot, M. and Ratjen, F. (1997) Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. *Pediatr. Pulmonol.,* **24**: 173-177.

Grasemann, H., Knauer, N., Buscher, R., Hubner, K., Drazen, J.M. and Ratjen, F. (2000) Airway nitric oxide levels in cystic fibrosis patients are related to

polymorphism in the neuronal nitric oxide synthase gene. *Am. J. Respir. Crit. Care Med.*, **162**: 2172-2176.

Green, S.J. and Nacy, C.A. (1993) Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthase. *Curr Opin infect dis.*, **6**:384-396.

Grisham, M.B., Jourd'heuil, D. and Wink, D.A. (1999) Nitric oxideI. physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *Am. J. Physiol.*, **276**: G315-G321.

Gross, S.S. and Wolin, M., S. (1995) Nitric oxide: Pathophysiological mechanisms. *Annu. Rev. Physiol.*, **57**: 737-769.

Guo, F H., Raeve, H.R., Rice, T.W., Stuehr, D.J., Thunnissen, F.B. and Erzurum, S.C. (1995) Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo. Proc. Natl. Acad. Sci. USA.*, **92**: 7809-7813.

Guo, F.H., Uetani, K., Haque, S.J., Williams, B.R., Dweik, R.A., Thunnissen, F.B., Calhoun, W. and Erzurum, S.C. (1997) Interferon gamma and interleukin 4 stimulate prolonged expression of inducible nitric oxide synthase in human airway epithelium through synthesis of soluble mediators. *J.Clin. Invest.*, **100**: 829-834.

Guo, F.H., Suzy, A.A., Comhair, A.A., Zheng, S., Zheng, S., Dweik, R.A., Eissa, N.T., Thomassen, M.J., Calhoun, W. and Erzurum, S.C. (2000) Molecular mechanisms of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. *J. Immunol.*, **164**: 5970-5980.

Haas, B., Kraut, J. and Marks, J., Zanker, S.C. and Castingnetti D. (1991) Siderophore presence in sputa of cystic fibrosis patients. *Infect. Immun.*, **59**: 3997-4000.

Halliwell, B. and Gutteridge, J.M.C. (1999) Free radicals in biology and medicine. Oxford university press. Thomson Press.

Hamid, Q., Springall, D.R., Riveros-Moreno, V., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S. and Polak, J.M. (1993) Induction of nitric oxide synthase in asthma. *Lancet,* **342**: 1510-1513.

Hassett, D.J., Ma, J.F., Elkins, J.G., McDermott, T.R., Oschesner, U.A., West, S.E.H., Huang, C.T., Fredericks, J., Burnett, S., Stewart, P.S., McFeters, G., Passador, L. and Iglewski, B.H. (1999a) Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes mediates biofilm susceptibility to hydrogen peroxide. *Mol. Microbiol.*, **34**:1082-1093.

Hassett, D.J., Elkins, J.G., Ma, J. and McDermott, T.R. (1999b) *Pseudomonas aeruginosa* biofilm sensitivity to biocides: use of hydrogen peroxide as model antimicrobial agent for examining resistance mechanisms. *Methods. Enzymol.*, **310**: 599-608.

Han, J., Luby-Phelp, S.K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falch, J.R., Whilte, M.A. and Brock, D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science,* **279**: 558-560.

Hertel, C., Schmidt, G., Fischer, M., Oellers, K. and Hammes W.P. (1998) Oxygen-dependent regulation of the expression of the catalase gene *katA* of *Lactobacillus sakei* LTH677. *Appl. Environ. Microbiol.*, **64**: 1359-1365.

Hengge-Aronis, R. (1999) Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli. Curr. Opin. Microbiol.*, **2**: 148-152.

Henrey, D.A., Mahenthiralingam, E., Vandamme, P., Coenye, T. and Spreet, D. (2001) Phenotypic methods for determining genomovar status of *Burkholderia cepacia*. *J. Clin. Microbiol.*, **39**: 1073-1078.

Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1987) Macrophage cytotoxicity role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science*, **253**: 473-476.

Hilman, B.C. and Constantinescu, M. (1999) Role of DNA testing in cystic fibrosis. *Lab. Med.*, **30**: 48-55.

Hoare, G.S., Marczin, N., Chester, A.H. and Yacoub, M.H. (1999) Role of oxidant stress in cytokine-induced activation of NF-κB in human aortic smooth muscle cells. *Am. J. Physiol.*, **277**: H1975-H1984.

Hoffman, R. (1996) Reactive nitrogen intermediates in immunology. *Methods*, **10**: 1-7.

Huasner, M. and Wuertz, S. (1999) High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Appl. Environ. Microbiol.*, **64**: 3710-3713.

Hughes, J.E., Strewart, J., Barclay, R. and Govan, J.R. (1997) Primimg of neutrophil respiratory burst activity by lipopolysaccharide from *Burkholderia cepacia*. *Infect. Immun.*, **65**: 4281-4287.

Hutchison, M.L. and Govan, R.W. (1999) Pathogenicity of microbes associated with cystic fibrosis. *Microes Infect.*, **1**: 1005-1014.

Imundo, L., Barasch, J., Prince, A. and Awqati, Q. (1995) Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. *Proc. Natl. Acad. Sci. USA.*, **92**: 3019-3023.

Ishihama, A. (1997) Adaptation of gene expression in stationary phase bacteria. *Curr. Opin. Genet. Dev.*, **7**: 582-588.

Isles, A., Maclusky, I., Corey, M., Gold, R., Rober, C., Fleming, P. and Levison, H. (1984) *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J. Pediatr.*, **104**: 206-210.

Jenkinson, C.P., Grody, W.W. and Cederbaum, S.D. (1996) Comparative properties of arginase. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.,* **114**: 107-132.

Jobin, C., Morteau, O., Han, D. and Sartor, R.B. (1998) Specific NF–κB blockade selectively inhibits tumour necrosis factor-α induced COX-2 but not constitutive COX-1 gene expression in HT-29 cells. *Immunolology*, **95**: 537-543.

Jørgensen, S., Skov, K.W. and Diderichsen, B. (1991) Cloning, sequence and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes. *J. Bacteriol.*, **173**: 559-657.

Jones, K.L., Bryan,T.W., Jinkins, P.A., Grisham, M.B., Owens, M.W., Milligan, S.A., Markwitz, B.A. and Robbins, K.A. (1998) Superoxide release from neutrophils causes reduction in nitric oxide gas. *Am. J. Physiol.*, 275: L1120-L1126.

Jourd'heuil, D., Jourd'heuil, F., Kutchukian, P.S., Musah, R.A., Wink, D.A. and Grisham, M.B. (2001) Reaction of superoxide and nitric oxide with peroxynitrite. *J. Biol. Chem.*, **276**: 28799-28805.

Karupiah, G., Xie, Q., Buller, M., Nathan, C., Duarte, C. and MacMicking, J.D. (1993) Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science,* **261**: 1445-1447.

Kasama, T., Strier, R.M., Lukacs, N.W., Burdick, M.D. and Kunkel, S.L. (1994) Regulation of neutrophil-derived chemokine expression by IL-10. *J. Immunol.,* **152**: 3559-3569.

Katsuwon, J. and Anderson, A.J. (1992) Characterization of catalase activities in a root-colonizing isolate of *Pseudomonas putida. Can. J. Microbiol.*, **38**: 10261032.

Kaufmann, S.H.E. (1993) Immunity to intracellular bacteria. *Annu. Rev. Immunol.*, **11**: 129-163.

Keig, P.M., Ingham, E. and Kerr, K.G. (2001) Invasion of human type II pneumocytes by *Burkholderia cepacia. Micro. Patho.,* **30**: 167-170.

Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science,* **245**: 1073-1080.

Kelley, T. and Drumm, M.L. (1998) Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. *J. Clin. Invest.,* **102**: 1200-1207.

Khan, T.Z., Wagener, J.S., Bost, T., Marinez, J., Accurso, F.J. and Riches, D.W.H. (1995) Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.*, **151**: 1075-1082.

Kharitonov, S.A. and Barnes, P.J. (2001) Exhaled markers of pulmonary disease. *Am. J. Respir. Crit. Care Med.,* **163**: 1693-1722.

Koarai, A., Ichinose, M.S.H., Yamagata, S., Hattori, T. and Shirato, K. (2000) Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by selective iNOS inhibitor, 1400W, in mice. *Pulm. Pharmacol. Ther.,* **13**: 267-275.

Kobzik, L.D., Bredt, D.S., Lowestein, C.J., Drazen, J., Gaston, B., Sugarbaker, D. and Stamler, J.S. (1993) Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am. J. Respir. Cell. Mol. Biol.,* **9**: 371-377.

Koichiro, A., Chee, C. B.E. and Gastron, B. (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. USA.*, **91**: 10089-10093.

Kolios, G., Brown, Z., Robson, R.L., Robertson, D.A.F. and Westwick, J. (1995) Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. *Br. J. Pharmacol.*, **116**: 2866-2872.

Kolios, G., Robertson, D.A.F., Jordan, N., Minty, D., Caput, D., Ferrara, P. and Westwick, J. (1996) Interleukin-8 production by human colon epithelial-cell line HT-29-modulation by interleukin-13. *Br. J. Pharmacol.,* **119**: 351-359.

Kolios, G., Rooney, N., Murphy, C.T., Robertson, D.A.F. and Westwick, J. (1998) Expression of inducible nitric oxide synthase activity in human colon epithelial cells: modulation by T lymphocyte derived cytokines. *Gut,* **43**: 56-63.

Konstan, M.W., Hilliard, K.A., Norvell, T.M. and Berger, M. (1994) Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am. J. Respir. Crit. Care Med.,* **150**: 448-454

Kooi, C.A., Cox, A., Darling, P. and Sokol, P.A. (1994) Neutralizing monoclonal antibodies to an extracellular *Pseudomonas cepacia* protease. *Infect. Immun.*, **62**: 2811-2817.

Knowles, R. and Moncada, S. (1994) Nitric oxide synthase in mammals. *Biochem. J.*, **298**: 249-258.

Kuo, P.C., Alfrey, E.J., Krieger, N.R., Abe, K.Y., Huie, P., Sibley, R.K. and Dafoe, D.C. (1997) Differential localization of allograft nitric oxide synthesis: comparison of liver and heart transplantation in the rat model. *Immunology*, **87**: 647-653.

Kwon, S., Newcomb, R.L. and George, C.S. (2001) Mechanism of synergistic cytokine-induced nitric oxide production in human epithelial cells. *Nitric oxide,* **5**: 534-546.

Lange, R. and Hengge-Arison, R. (1991) Growth phase regulated expression of bolA and morphology of stationary–phase *Escherichia coli* cells are controlled by a novel sigma factor σ^s. *J. Bacteriol.*, **173**: 4474-4481.

Lefebre, M.D. and Volvano, M.A. (2001) *In vitro* resistance of *Burkholderia cepacia* complex isolates to reactive oxygen species in relation to catalase and superoxide dismutase production. *Microbiology,* **147**: 97-109.

Lentsch, A.B., Shnley, T.P., Sarma, V. and Ward, P.A. (1997) *In vivo* suppression of NF-kappa B and preservation of I kappa B α by interleukin-10 and interleukin-13. *J. Clin. Invest.*, **100**: 2443-2449.

Lentsch, A.B., Czermak, B., J., Jordan, J. and Ward, P., A. (1999) Regulation of acute lung inflammatory injury by endogenous IL-13. *J. Immunol.*, **162**: 1071-1076.

Lessie, T.G., Hendrickson, B., Manning, D. and Devereux, R. (1996) Genomic complexity and plasticity of *Burkhoderia cepacia*. *FEMS Microbiol. Lett.*, **144**: 117-128.

Lewenza, S., Conway, B., Greenberg, B. and Sokol, P.A. (1999) Quorum sensing in *Burkholderia cepacia*: identification of the LuxR1 homologs CepRI. *J. Bacteriol.*, **181**: 748-751.

Liew, F.Y., Severn, A., Millott, S., Schmidt, J., Salter, M. and Moncada, S. (1991) A possible novel pathway of regulation by murine T helper type-2 (Th2) cells of a Th1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. *Eur. J. Immunol.*, **21**: 2489-2494.

Linnane, S.J., Keatings, V.M., Costello, C.M., Moynihan, J.B., O'Corrnor, C.M., Fitzgerald, M.X. and Mclonghin, P. (1998) Total sputum nitrate plus nitrite is raised during acute pulmonary infection in cystic fibrosis. *Am. J. Respir. Crit. Care Med.*, **158**: 207-212.

Loewen, P.C. (1984) Isolation of catalase-defecient *Escherichia coli* mutants and genetic mapping of *katE*, a locus that affects catalase activity. *J. Bacteriol.*, **157**: 622-626.

Loewen, P.C. and Triggs, B.L. (1984) Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia coli*. *J. Bacteriol.*, **160**: 668-675.

Loewen, P.C., Switala, J. and Tiggs-Raine, B. (1985a) Catalase HPI and HPII in *Escherichia coli* are induced independently. *Arch. Biochem. Biophys.*, **243**: 144-149.

Loewen, P.C., Triggs-Raine, B. and George, C.S. (1985b) Genetic mapping of *katG*, a locus that affects synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in *Escherichia coli*. *J. Bacteriol.*, **162**: 661-667

Loewen, P.C. and Hengge-Aronis, R. (1994) The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Ann. Rev. Microbiol.*, **48**: 53-80.

Loewen, P.C. (1996) Probing the structure of catalase HPII of *Eschrichia coli*-a review. *Gene*, **179**: 39-44.

Loewen, P.C., Hu, B., Strutinsky, J. and Sparling, R. (1998) Regulation of in the rpoS regulon of *Escherichia coli*. *Can. J. Microbiol.*, **44**: 707-717.

Loprasert, S., Vattanaviboon, P., Praituan, W., Chamnongpol, S. and Mongkolsuk, S. (1996) Regulation of the oxidative stress protective enzymes, catalase and superoxide dismutase in *Xanthomonas*. *Gene*, **179**: 33-37.

Lutter, E., Lewenza, S., Dennis, J.J., Visser, M.B. and Sokol, P.A. (2001) Distribution of quorum-sensing genes in *Burkholderia cepacia* complex. *Infect. Immun.*, **69**: 4661-46666.

Ma, M. and Eaton, J.W. (1992) Multicellular oxidant defence in unicellular organisms. *Proc. Natl. Acad. Sci. USA.*, **89**: 7924-7928.

MacMicking, J., Xie, W. and Nathan, C. (1997) Nitric oxide and macrophage function. *Annu. Rev. Immunol.*, **15**: 323-330.

Mahenthiralingam, E., Simpson, D.A. and Speert, D.P. (1997) Identification and characterisation of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. *J. Clin. Microbiol.*, **38**: 910-913.

Manna, S.K. and Aggarwal, B.B. (1998) IL-13 suppresses TNF-induced activation of nuclear factor- κB, activation protein-1 and apoptosis. *J. Immunol.,* **161**: 2863-2872.

Marolda, C.L., Hauroder, B., John, M.A., Michel, R. and Valvano, M.A. (1999) Intracellular survival and saprophytic growth of isolates from *Burkholderia cepacia* Complex in free living amoebae. *Microbiology,* **145**: 1509-1517.

Martin, D.W. and Mohr, C.D. (2000) Invasion and intracellular survival of *Burkholderia cepacia. Infect. Immun.*, **68**: 24-29.

Martin, E., Nathan, C. and Xie, Q.W. (1994) Role of interferon regulatory factor-1 in induction of nitric oxide synthase. *J. Exp. Med.*, **180**: 977-984.

Massengale, A.R.D., Quinn, F., Jr. Yankaskas, J.R., Weissmann, D.M., Cuff, C. and Aronoff, S.C. (1999) Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.,* **20**: 1073-1080.

Matsui, H., Grubb, B.R. and Tarran, R. (1999) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell*, **95**: 1005-1015.

McKenney, D. and Allison, D.G. (1995) Effects of growth rate and nutrient limitation on virulence factor production in *Burkholderia cepacia. J. Bacteriol.*, **177**: 4140-4143.

McKenney, D., Brown, K.E. and Allison, D.G. (1995) Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. *J. Bacteriol.,* **177**: 6989-6992.

Mellot, J.K., Nick, H.S., Waters, M.F., Billiar, T.R., Geller, D.A. and Chesrown, S.E. (2001) Cytokine-induced changes in chromatin structure and in vivo footprints in the inducible NOS promoter. *Am. J. Physiol.,* **280**: L390-L399.

Melnikov, A., Zaborina, O., Dhiman, N., Prabhakar, B.S., Chakrabarty, A.M. and Hendrickson, W. (2000) Clinical and environmental isolates of *Burkholderia cepacia* exhibit differential cytotoxicity towards macrophages and mast cells. *Mol. Biol.*, **36**: 1481-1493.

Meng, Q.H., Springall, D.R. and Bishop, A.E. (1998) Lack of inducible nitric oxide synthase in bronchial epithelium: possible mechanism of susceptibility to infection in cystic fibrosis. *J. Pathol.,* **184**: 323-331.

Meyer, K.C. and Sharma, A. (1997) Regional variability of lung inflammation in cystic fibrosis. *Am. J. Respir. Crit. Care Med.,* **156**: 1536-1540.

Miles, A.A. and Misera, S.S. (1938) The estimation of the bactericidal power of the blood. *J. Hyg.,* **38**:732 - 749.

Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M. and Currie, M.G. (1993) A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.,* **214**: 11-16.

Moncada, S. and Higgs, A. (1993) The L-arginine-nitric oxide pathway. *N Engl J. Med.,* **30**: 2002-2012.

Mongkolsuck, S., Loprasert, S., Vattanaviboon, P., Chanvanichayachai, C., Chamnongpol, S. and Supsamran, N. (1996). Heterologous growth phase- and temperature-dependent expression and H_2O_2 toxicity protection of a superoxide-

inducible monofunctional catalase gene from *Xanthomonas oryzae* pv. *Oryzae. J. Bacteriol.*, **178**: 3578-3584.

Mongkolsuck, S., Loprasert, S., Vattanaviboon, P., Chanvanichai, C., Chamnongpol, S. and Supsamran, N. (1996) Heterologous growth phase- and temperature-dependent expression and H_2O_2 toxicity protection of a superoxide-inducible monofunctional catalase gene from *Xanthomonas oryzae. J. Bacteriol.*, **178**: 3578-3584.

Morcillo, E., J., Esrela, J. and Cortijo, J. (1999) Oxidative stress and pulmonary inflammation pharmacological intervention with antioxidatnts. *Pharmacol. Res.*, **40**: 393-404.

Mori, M. and Gotoh, T. (2000) Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem. Biophys. Res. Commun.*, **275**: 715-719.

Moskovitz, J., Rahman, M.A., Stassman, J., Yancey, S.O., Kushner, S.R., Brot, N. and Weissbach, H. (1995) *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. *J. Bacteriol.*, **177**: 502-507.

Moskovitz, J., Berlett, B.S., Poston, J.M. and Stadtman, E.R. (1997) The yeast peptide-methionine sulfoxide reductase functions as an antioxidant *in vivo. Proc. Natl. Acad. Sci. USA.*, **94**: 9585-9589.

Moss, R.B., Bocian, R.C., Hsu, Y.P., Dong, Y.J., Kemna, M., Wei, T. and Gardner, P. (1996) Reduced IL-10 secretion by $CD4^+$ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). *Clin. Exp. Immunol.*, **106**: 374-388.

Moss, R.B., Hsu, Y.P. and Olds, L. (2000) Cytokine dysregulation in activated cystic fibrosis (CF) peripheral lymphocytes. *Clin. Exp. Immunol.,* **120**: 518-525.

Muhlebach, M.S., Stewart, P.W., Margaret, W., Leicgh, W. and Noah, T.L. (1999) Quantitation of inflammatory response to bacteria in young cystic fibrosis and control patients. *Am. J. Crit. Care Med.,* **160**: 186-191.

Muijsers, R.B., tenHacken, N.H., vanArk, I.V., Folkerts, G., Nijkamp, F.P. and Ponstma, D.S. (2001) L-arginine is not the limiting factor for nitric oxide synthesis by human alveolar macrophages *in vitro*. *Eur. Resp. J.,* **18**: 667-671.

Mulvey, M.R., Sorby, P.A., Triggs-Raine, B.L. and Loewen, P.C. (1988) Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. *Gene,* **73**: 337-345.

Mulvey, M.R., Switala, J., Borys, A. and Loewen, P.C. (1990) Regulation of *katE* and *katF* in *Escherichia coli*. *J. Bacteriol.*, **172**: 6713-6720.

Munder, M., Eichmann, K., Moran, J.M., Centeno, F., Soler, G. and Modellel, M. (1999) Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dentritic cells. *J. Immunol.,* **163**: 3771-3776.

Murphy, C. and Newsholme, P. (1998) Importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production. *Clin. Sci.,* **95**: 397-407.

Nakamura, H., Yoshimura, K., McElvaney, N.G. and Crystal, R.G. (1992) Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J. Clin. Invest.*, **89**: 1478-1484.

Nakazawa, T., Yamada, Y. and Ishibashi, M. (1987) Characterization of hemolysin in extracellular products of *Pseudomonas cepacia*. *J. Clin. Microbiol.*, **25**: 195-198.

Nathan, C.F. and Hibbs, J.B. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.*, **3**: 65-70.

Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.*, **6**: 3051-3064.

Nathan, C. and Xie, Q.W. (1994) Nitric oxide synthase: roles, tolls and controls. *Cell*, **78**: 915-918.

Nathan, C.F. and Shiloh, M.U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Natl. Acad. Sci USA.*, **16**: 8841-8848.

Nicolson, A.G., Haites, N.E., McKay, N.G., Wilson, H.M., MacLeod, A.M. and Benjamin, N. (1993) Induction of nitric oxide synthase in human mesangial cells. *Biochem. Biophys. Res. Commun.*, **193**: 1269-1274.

Nussler, A.K., Di Silvio, M., Billar, T.R., Hoffman, R.A., Geller, D.A., Selby, R., Madariaga, J. and Simmons, R.L. (1992) Stimulation of nitric oxide pathway in human hepatocytes by cytokines and endotoxin. *J. Exp. Med.*, **176**: 261-264.

Oceandy, D., McMorran, B.J., Smith, S.N., Schreibe, R., Kunzelmann, K., Alton, E.W., Hume, D.A. and Waiwright, B.J. (2002) Gene complementation of airway epithelium in the cystic fibrosis mouse is necessary and sufficient to correct the pathogen clearance and inflammatory abnormalities. *Hum. Mol. Genet.*, **11**: 1059-1067.

Olson, E.R. (1993) Influence of pH on bacterial gene expression. *Mol. Microbiol.*, **8**: 5-14.

Pacelli, R., Wink, D.A., Cook, J.A., Krishana, M.C., DeGraff, W., Friedman, N., Tosokos, M., Samuni, A. and Mitchell, J.B. (1995) Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli. J. Exp. Med.*, **182**: 1469-1479.

Pahan, K., Raymond, J.R. and Singh, I. (1999) Inhibition of phosphatidylinositol 3-kinase induces nitric-oxide synthase in lipopolysaccharide- or cytokine-stimulated C6 glial cells. *J. Biol. Chem.*, **274**: 7528-7538.

Palfreyman, R.W., Watson, M.L., Eden, C. and Smith, A.W. (1997) Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia cepacia* products. *Infect. Immun.*, **65**: 617-622.

Park, Y.C., Lee C.H., Kang, H.S., Chung, H.T. and Kim, H.D. (1997) Wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, enhances LPS-induced NO production from murine peritoneal macrophages. *Biochem. Biophys. Res. Commun.*, **240**: 692-696.

Parsons, Y.N., Glendinning, K.J., Thronton, V., Hales, B.A., Hart, C.A. and Winstanley, C. (2001) A putative type III secretion gene cluster widely distributed in the Burkholderia cepacia complex but absent from genomovar I. *FEMS Microbiol. Lett.*, **203**: 103-108.

Pechovsky, D.V., Zissel, G., Goldmann, T., Einhaus, M., Taube, C., Magnussen, H., Schlaak, M. and Muller-Quernheim (2001) Different pattern of NOS2 and NOS3 mRNA expression in human A549 cells and primary cultured alveolar epithelial cells type II. *Am. J. Physiol.*, **282**: L684-692.

Pendino, K.J., Laskin, J.D., Shuler, R.L., Punjabi, C.J. and Laskin, D.L. (1993) Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of pulmonary irritant is associated with increased expression of nitric oxide synthase. *J. Immunol.*, **151**: 7196-7205.

Pier, G.B. (2000) Role of cystic fibrosis transmembrane conductance regulator in innate immunity to *Pseudomonas aeruginosa* infections. *Proc. Natl. Acad. Sci. USA.*, **97**: 8822-8828.

Pilewski, J.M. and Frizzell, R.A. (1999) Role of CFTR in airway disease. *Physiol. Rev.*, **79**: S215-S255.

Pitt, B.R. (2001) CFTR trafficking and signaling in respiratory epithelium. *Am. J. Physiol.*, **281**: L13-L15.

Pitt, B.R. and Croix, M.St. (2002) Complex regulation of iNOS in lung. *Am. J. Respir. Cell Mol. Biol.*, **26**: 6-9.

Poole, R.K. and Hughes, M.N. (2000) New function for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol. Microbiol.*, **36**: 775-783.

Purdy, D., Cawthraw, S. and Dickinson, J.H. (1999) Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in Campylobacter survival and colonization. *Appl. Environ. Microbiol.*, **65**: 2540-2546.

Que, L.G., George, S.E., Gotoh, T., Mori, M. and Huang, Y. T. (2002) Effects of arginase isoforms on NO production by nNOS. *Nitric Oxide,* **6**: 1-8.

Quinton, P.M. (1999) Physiological basis of cystic fibrosis: a historical prospective. *Physiol. Rev.*, **79**: S3-S22.

Rahman, I. and MacNee, W. (1998) Role of transcription factors in inflammatory lung disease. *Thorax,* **53**: 601-612.

Rameh, L.E. and Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.,* **274**: 8347-8350.

Rashid, M.H., Rumbaugh, K., Passador, L., Davies, D.G., Hamood, A., Iglewski, B.H. and Kornberg, A. (2000) Polyphosphate kinase is essential for biofilm development, quorum sensing and virulence of *Pseudomonas aeruginosa. Proc. Natl. Acad. Sci USA.,* **97**: 9636-9641.

Repine, J., Bast, A. and Lankhorst, I. (1997) Oxidative stress in chronic obstructive pulmonary disease. *Am. J. Crit. Care Med.,* **156**: 341-357.

Revets, H., Vandamme, P., Van Zeebroeck, A., De Boeck, K., Struelens, M.J., Verhaegen, J., Ursi, J.P., Verschraegen, G., Franckx, H., Malfroot, A., Dab, I. and Lauwers, S. (1996) *Burkholderia (Pseudomonas) cepacia* and cystic fibrosis: an epidemiology in Belgium. *Acta. Clin. Belg.,* **51**: 222-230.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H. and Hoiby, N. (2001) N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology,* **147**: 3249-3262.

Riordan, J., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S. and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science,* **245**: 1066-1073.

Robbe-Saule, V., Coynault, C., Ibanz-Ruiz, M., Hermant, D. and Norel, F. (2001) Identification of a non-haem catalase in Salmonella and its regulation by RpoS (σ^S). *Mol. Microbiol.*, **39**: 1533-1545.

Robbins, R. Barnes, P.J., Springall, D.R., Warren, J.B., Know, O.J., Buttery, L.D.K., Wilson, A.J., Geller, D.A. and Polak, J.M. (1994) Expression of inducible nitric oxide in human lung epithelial cells. *Biochem. Biophys. Res Comm.,* **203**: 209-218.

Robbins, R.A., Hadeli, K., Nelson, D., Sato, E. and Hoyt, J.C. (2000) Nitric oxide, peroxynitrite and lower respiratory tract inflammation. *Immunolology,* **48**: 217-221.

Rocha, E.R. and Smith, C.J. (1997) Regulation of *Bacteriodes fragilis katB* mRNA by oxidative stress and carbon limitation. *J. Bacteriol.*, **179**: 7033-7039.

Rommens, J.M., Iannuzzi M.C., Kerem B., Drumm M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N. and et al. (1989) Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science,* **245**: 1059-1065.

Russell, J.P., Diamond, G., Tarver, A.P., Scanlin, T.F. and Bevins, C.L. (1996) Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumour necrosis factor-α. *Infect. Immun.*, **64**: 1565-1568.

Rutschman, R., Lang, R., Hesse, M., Ihle, N.N., Wynn, T.A. and Murry, P. J. (2001) Cutting edge: Stat6-dependent substrate regulates nitric oxide production. *J. Immunol.,* **166**: 2173-2177.

Saini, L.S., Galsworthy, S.B., John, M.A. and Valvano, M.A. (1999) Intracellular survival of *Burkholderia cepacia* complex isolated in the presence of macrophage cell activation. *Microbiology,* **145**: 3465-3475.

Sajjan, U.S., Sun, R., Goldstein, R. and Forstner, F.J. (1995) Cable (*cbl*) type II pili of cystic fibrosis-associated *Burkholderia* (*Pseudomonas*) *cepacia*: nucleotide sequence of the *cblA* major subunit pilin gene and novel morphology of the assembled appendage fibers. *J. Bacteriol.*, **177**: 1030-1038.

Sambrook, J., Fitsch, F.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour, N.Y.: Cold Spring Harbour Laboratory Press.

Sanchez-Madrid, F. and Del Pozo, M.A. (1999) Leukocyte polarization in cell migration and immune interactions. *EMBO J.,* **18**: 501-511.

Sanders, N.N., De Smedt, S., VanRompaey, E., Simoens, P., DeBaets, F. and Demeester, J. (2000) Cystic fibrosis sputum. *Am. J. Respir. Crit. Care Med.,* **162**: 1905-1911.

Saura, M., Martinez-Dalmau, R., Minty, A., Perez-Sala, D. and Lamas, S. (1996) Interleukin-13 inhibits inducible nitric oxide synthase expression in human mesangial cells. *Biochem. J.,* **313**: 641-646.

Sawa, T., Corry, D.B., Gropper, M.A., Oharra, M., Kurahashi, K. and Wiener-Kornish, J.P. (1997) IL-10 improves lung injury and survival in *Pseudomonas aeruginosa* pneumonia. *J. Immunol.*, **159**: 2858-2866.

Schellhorn, H.E. (1995) Regulation of hydroperoxidase (catalase) expression in *Escherichia coli. FEMS Microbiol. Lett.*, **131**: 113-119.

Schellhorn, H.E. and Hassan, H.M. (1988) Transcriptional regulation of KatE in *Escherichia coli* K-12. *J. Bacteriol.*, **170**: 4286-4292.

Schmit, H.H.W. and Walter, U. (1994) NO at work. *Cell,* **78**: 919-925.

Schneider, T.Y. and Dy, M. (1985) The role of arginase in the immune response. *Immunol. Today,* **6**: 136-140.

Shaw, D.I., Poxton, I.R. and Govan, R.W. (1995). Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide. *FEMS Immunol. Med. Microbiol.,* **11**: 99-106.

Shaw, M., Cohen, P. and Alessi, D.R. (1998) The activation of protein kinase B by H_2O_2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem. J.,* **336**: 241-246.

Shimomura, H., Matsuura, M., Saito, S., Hirai, Y., Isshiki, Y. and Kawahara, K. (2001) Lipopolysaccharide of *Burkholderia cepacia* and its unique character to stimulate murine macrophages with relative lack of interleukin-1β-inducing ability. *Infect. Immun.,* **69**: 3663-3669.

Simon, R.H., DeHart, P.D. and Todd3rd. , R.F. (1986) Neutrophil-induced injury in rat pulmonary alveolar epithelial cells. *J. Clin. Invest.*, **78**: 1375-1386.

Singh, S.P., Schaefer, A.L., Parsek,, M.P., Moninger, T.O., Welsh, M.J. and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature,* **407**: 762-764.

Smith, A.W., Green, J., Eden, S. and Watson, M.L. (1999) Nitric oxide-induced potentiation of the killing of *Burkholderia cepacia* by reactive oxygen species: implications for cystic fibrosis. *J. Med. Microbiol.*, **48**: 419-423.

Smith, J., Travis, S., Greenberg, E. and Welsh, M. (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell,* **85**: 229-236.

Soltys, J., Bonfield, T., Chmiel, J. and Berger, M. (2002) Functional IL-10 deficiency in the lung of cystic fibrosis (*cftr*-/-) and IL-10 knockout mice causes increased expression and function of B7 costimulatory molecules on alveolar macrophages. *J. Immunol.*, **168**: 1903-1910.

Sonoda, Y., Wantanabe, S., Matsumoto, Y., Aizu-Yokota, E. and Kasahara, T. (1999) FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line. *J. Biol. Chem.*, **274**: 10566-10570.

Sonoki, T., Nagazaki, A., Gotoh, T., Masaki, T., Takeya, M., Matsuzaki, H. and Mori, M. (1997) Co-induction of nitric oxide synthase and arginase I in cultured rat peritoneal macrophages *in vivo* by lipopolysaccharide. *J. Biol. Chem.*, **7**: 3689-3693.

Sozzani, P., Cambon, C., Vita, N., Seguelas, M.H., Caput, D., Ferrara, P. and Pipy, B. (1995) Interleukin-13 inhibits protein–kinase C-triggered respiratory burst in human monocytes – role of calcium and cyclic-AMP. *J. biol. Chem.,* **270**: 5084-5088.

Speert, D.P., Bond, M., Woodman, R.C. and Curnutte, J.T. (1994) Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of non-oxidative killing by neutrophils in host defence. *J. Infect. Dis.,* **170**: 1524-1531.

Speert, D.P., Henry, D., Vandamme, P., Corey, M. and Mahenthiralingam, E. (2002) Epidemiology of *Burkholderia cepacia* in patients with cystic fibrosis. *Can. Emerg. Infect. Dis.,* **8**: 181-187.

Standiford, T.J., Kunkel, S.L., Bashe, M.A., Chensue, S.W., Lynch III, J.P., Galen, B.T., Westwick, J. and Strieter, R.M. (1990) Interleukin-8 gene expression by a pulmonary epithelial cell line. *J. Clin. Invest.*, **86**: 1945-1953.

Stephens, L.R., Jackson, T.R. and Hawkins, P.T. (1993) Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-triphosphate: a new intracellular signaling system? *Biochem. Biophys. Acta.*, **1179**: 27-75.

St.John, G., Nathan, B., Ruan, J., Erdjument-Bromage, H., Tempst, P., Weissbach, H. and Nathan, C. (2001) Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. *Proc. Natl. Acad. Sci. USA.*, **98**: 9901-9906.

Storz, G., Tartagila, L.A. and Ames, B.N. (1990) Transcriptional regulation of oxidative stress inducible genes: direct activation by oxidation. *Science,* **248**: 189-194.

Storz, G. and Tartagila, L.A. (1992) OxyR: a regulator of antioxidant genes. *J. Nutr.*, **122**: 627-630.

Straus, D.C., London, M. and Huston, J.C. (1992) Inhibition of rat alveolar macrophage phagocytic function by a *Pseudomonas cepacia* lipase. *J. Med. Microbiol.*, **37**: 335-340.

Stuehr, D.J. and Griffith, O.W. (1992) Mammalian nitric oxide synthesis. *Adv. Enzymol.*, **65**: 287-346.

Stuehr, D.J. (1999) Nitric oxide synthesis and breakdown. *Biochem. Biophys. Acta.*, **1411**: 217-224.

Sun, L., Jiang, R.Z., Steinbach, S., Holmes, A., Campanelli, C., Fosstner, J., Sajjan, U., Tan, Y., Riley, M. and Goldstein, R. (1995) The emergence of a highly transmissible lineage of *cbl+ Pseudomonas (Burkholderia) cepacia* causing CF center epidemics in North America and Britain. *Nat. Med.,* **1**: 661-666.

Swift, S., Karlyshev, A.V., Fish, L., Salmond, G.P., Stewart, G.S. and Williams, P. (1997) Quorum sensing in population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.,* **21**: 214-219.

Switala, J., Triggs-Raine, B. and Loewen, P.C. (1990) Homology among bacterial catalase genes. *Can. J. Microbiol.,* **36**: 728-731.

Tablan, O.C., Chrba, T.L., Schidlow, D.V., White, J.W., Hardy, K.A., Gilligan, P.H., Morgan, W.M., Carson, L.A., Martone, W.J., Jason, J.M. and Jarvis, W.R. (1985) *Pseudomonas cepacia* colonization in patients with cystic fibrosis: risk factors and clinical outcomes. *J. Pediatr.,* **107**: 382-387.

Tao, H., Bausch, C., Richmond, G., Blatter, F.R. and Conway, T. (1999) Functional genomic: expression analysis of *Escherichia coli* growing on minimal and rich media. *J. Bacteriol.,* **181**: 6425-6440.

Taylor, B.S., Alarcon, L.H. and Billiar, T.R. (1998) Inducible nitric oxide synthase in the liver: Regulation and function. *Biochemistry,* **63**: 766-781.

Thomas, S., Kharitonov, S.A., Scott, M.E., Hodson, M.E. and Barnes, P.J. (2000) Nasal and exhaled nitric oxide is reduced in adult patients with cystic fibrosis and does not correlate with cystic fibrosis genotype. *Chest,* **117**: 1085-1089.

Tipper, J.L., Ingham, E., Gove, J.H., Todd, N.J. and Kerr, K.G. (1998) Survival and multiplication of *Burkholderia cepacia* within respiratory epithelial cells. *Clin. Microbiol. Infect.,* **4**: 450-459.

Toker, A. (2000) Protein kinases as mediators of phosphoinositide 3-kinas signaling. *Mol. Pharmacol.*, **57**: 652-658.

Tomich, M., Herfest, C.A., Golden, J.W. and Mohr, C.D. (2002) Role of flagella in host cell invasion by *Burkholderia cepacia*. *Infect. Immun.*, **70**: 1799-1806.

Triggs-Raine, B. and Loewen, P.C. (1987) Physical characterization of KatG, encoding catalase HPII of *Escherichia coli*. *Gene,* **52**: 121-128.

Tummler, B. and Kiewitz, C. (1999) Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. *Mol. Med. Today,* **5**: 351-358.

Tyler, S.D., Rozee, K.R. and Johnson, W.M. (1996) Identification of IS1356, a new insertion sequence and its association with IS402 in epidemic strains of *Burkholderia cepacia* infecting cystic fibrosis patients. *J. Clin. Microbiol.*, **34**: 1610-1616.

Uetani, K., Arroliga, M.E. and Erzurum, S. (2001) Double-stranded RNA dependence of nitric oxide synthase 2 expression in human bronchial epithelial cell lines BET-1A and BEAS-2B. *Am. J. Respir. Cell Mol. Biol.*, **24**: 720-726.

Van Delden, C. and Iglewiski, B.H. (1998) Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg. Infect. Dis.*, **4**: 551-560.

van der Vliet, A.V., Eiserich, J., P., Shigenaga, M., K. and Cross, C., E. (1999) Reactive nitrogen species and tyrosine nitration in the respiratory tract. *Am. J. Respir. Crit. Care Med.*, **160**: 1-9.

van der Vliet, A.V. and Cross, C.C. (2000) Oxidants, nitrosants and the lung. *Am. J. Respir. Crit. Care Med.*, **109**: 398-421.

Vanhaesebroeck, B. and Waterfield, M.D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. *Exp. Cell. Res.*, **253**: 239-254.

Vanhaesebroeck, B. and Alessi, D.R. (2000) The PI3K –PDK-1 connection: more than just a road to PKB. *Biochem. J.*, **346**: 561-576.

Vankeerberghen, A., Cuppens, H. and Cassiman, J. (2001) The cystic fibrosis transmembrane conductance regulator: an intriguing protein with plieotropic function. *J. Cystic Fibrosis,* **1**: 13-19.

Vattanaviboon, P. and Mongkolsuk, S. (2000) Expression analysis and characterization of the mutant of a growth-phase and starvation-regulated monofunctional catalase gene from *Xanthomonas campestris pv. phaseoli. Gene,* **241**: 259-265.

Velsor, L.W., Van Heeckeren, A. and Day, B. J. (2001) Antioxidant imbalance in the lung of cystic fibrosis transemembrane conductace regulator protein mutant mice. *Am. J. Physiol.*, **281**: L31-L38.

Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.*, **269**: 5241-5248

von Ossowski, I., Mulvey, M.R., Leco, P.A., Borys, A. and Loewn, P.C. (1991) Nucleotide sequence of *Escherichia coli katE*: which encodes catalase HPII. *J. Bacteriol.*, **173**: 514-520.

Wang, W. W., Jenkinson, C.P. and Griscavage, J.M. (1995) Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. *Biochem. Biophys. Res. Commun.*, **210**: 1009-1016.

Wang, Q.D., Kim, S.H., Wang, X.F. and Evers, B.M. (2000) Activation of NF-κB Binding in HT-29 Colon Cancer Cells by Inhibition of Phosphatidylinositol 3-Kinase *Biochem. Biophys. Res. Commun.*, **273**, 853-858.

Watnick, P. and Kolter, R. (2000) Biofilm, city of microbes. *J. Bacteriol.*, **182**: 2675-2679.

Weber, A.J., Bryan, R., Saba, S. and Prince, A. (2001) Activation of NF-κB in airway epithelial cells is dependent on CFTR trafficking and Cl^- channel function. *Am. J. Physiol.*, **281**: L71-L78.

Welham, M.J., Learmonth, L., Bone, H. and Scrahder, J. (1995) Interleukin-13 signal-transduction in lymphohematopoietic cells-similarities and differences in signal-transduction with IL-4 and insulin. *J. Biol. Chem.*, **270**: 12286-12296.

Welsh, M.J. and Ramsey B.W. (1998) Research on cystic fibrosis, A journey from the heart house. *Am. J. Respir. Crit. Care Med.*, **157**: S148-154.

West, S.E.H. and Iglewski, B.H. (1988) Codon usage in *Pseudomonas aeruginosa. Nucleic Acid Res.*, **16**: 9323-9335.

Whittle, B. J. R. (1995) Nitric oxide in physiology and pathology. *Histochem. J.* **27**: 727-735.

Wilks, K.E., Dunn, K.P.L., Farrant, J.L., Reddin. K.M., Gorringe, A.R., Langford, P.R. and Koll, J.S. (1998) Periplasmic superoxide dismutase in meningococcal pathogenicity. *Infect. Immun.*, **66**: 213-217.

Williams, P., Camara, M., Hardman, A., Swift, S., Milton, D., Hope, V.J., Winzer, K., Middeton, B., Pritchard, D.I. and Bycroft, B.W. (2000) Quorum

sensing and population-dependent control of virulence. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, **355**: 667-680.

Wilmott, R.W., Kassab, J.T., Killian, P.L., Benjamin, W.R. and Douglas, S.D. (1990) Increased levels of interleukin-1 in bronchioalveolar washings from children with bacterial pulmonary infections. *Am. Rev. Respir. Dis.*, **142**: 365-368.

Winstanley, C. (2001) A putative type III secretion gene cluster is widely distributed in the *Burholderia cepacia* complex but absent from genomvar I. *FEMS Microbiol. Lett.* **203**: 103-108.

Wonjnarowski, C., Fischer, T., Hofbauer, E., Grabner, C., Mosgoeller, W., Eichler, I. and Ziesche, R. (1999) Cytokine expression in bronchial biopsies of cystic fibrosis patients with and without acute exacerbation. *Eur. Respir. J.*, **14**: 1136-1144.

Wood, A.J.J. and Editor, M.D. (1996) Management of pulmonary disease in patients with cystic fibrosis. *N. Engl. J. Med.*, **335**: 179-188.

Wood, N.J. and Sorensen, J. (2001) Catalase and superoxide dismutase activity in ammonia-oxidising bacteria. *FEMS Microbial. lett.*, **38**: 53-58.

Woodbury, W., Spencer, A.K. and Stahmann, M.A. (1971) An improved procedure using ferricyanide for detecting calatalse isozymes. *Anal. Biochem.*, **44**: 301-305.

Wright, K., Ward, S.G., Kolios, G. and Westwick, J. (1997) Activation of phosphatidylinositol 3-kinase by interleukin-13. *J. Biol. Chem.*, **272**: 12626-12633.

Wright, K., L. and ward, S., G. (2000) Interaction between phosphatidylinositol 3-kinase and nitric oxide: explaining the paradox. *Mol. Cell Biol. Res. Commun.*, **4**: 137-143.

Wu, G., Sidney, M. and Morris, JR. (1998) Arginine metabolism: nitric oxide and beyond. *Biochem. J.*, **336**:1-17.

Wymann, M.P., Bulgarelli-Leva, G., Zvelebil, M.J., Pirola, L., Vanhasebrooeck, M., Waterfield, M.D. and Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. biol.*, **16**: 1722-1733.

Wymann, M.P. and Pirola, L. (1998) Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta.*, **1436**: 127-150.

Xie, Q. and Nathan, C (1994) The high-output nitric oxide pathway: role and regulation. *J. Leukoc. Biol.*, **56**: 576-582.

Xu, K.D., Stewart, P.S., Xia, F., Huang, C.T and McFeters, G.A. (1998) Physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol.* **64**: 4035-4039.

Xu, K.D., McFeter, G.A. and Stewart, P.S. (2000) Biofilm resistance to antimicrobial agents. *Microbiology,* **146**: 2481-2493.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. (1992) Proposal of *Burkholderia* gen. Nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia. Microbiol. Immunol.*, **36**: 1251-1275.

Yessel, H., De Waal Malefyt, R., Roncarolo, M.G., Abrams, J.S., Lahesmaa, R., Spits, H. and de Vries, J.E. (1992) IL-10 is produced by subsets of human $CD4^+$ T cell clones and peripheral blood T cells. *J. Immunol.,* **149**: 2378-2384.

Zahm, J.M., Gaillard, D., Dupuit, F., Hinnrasky, J., Porteous, D., Dorin, J.R. and Puchelle, E. (1997) Early alterations in airway mucociliary clearance and inflammation of lamina propia in CF mice. *Am. J. Physiol.*, **272**: C853-859.

Zamocky, M. and Koller, F. (1999) Understanding the structure and function of catalase: clues from molecular evolution and *in vitro* mutagenesis. *Prog. Biophys. Mol. Biol.,* **72**: 19-66.

Zanetti, F., De Luca, G. and Stampi, S. (2000) Recovery of *Burkholderia pseudomallie and Burkholderia cepacia* from drinking water. *Int. J. Food Microbiol.*, **59**: 67-72.

Zhang, P., Summer, G.J. and Nelson, S., (2000) Innate immunity and pulmonary host defence. *Immunol. Rev.,* **173**: 39-51.

Zhu, L., Gunn, C. and Beckman, J.S. (1992) Bactericidal activity of peroxynitrite. *Arch. Biochem. Biophys.,* **298**: 452-457.

Zughaier, S.M., Ryley, H.C. and Jackson, S.K. (1999a) A melanin pigment purified from an epidemic strain of *Burkholderia cepacia* attenuates monocytes respiratory burst activity by scavenging superoxide anion. *Infect. Immun.,* **67**: 908-913.

Zughaier, S.M., Ryley, H.C. and Jackson, S.K. (1999b) Lipopolysaccharide (LPS) from *Burkholderia cepacia* is more active than LPS from *Psuedomonas aeruginosa* and *Stenotrophomonas maltophilia* in stimulating tumor necrosis factor alpha from human monocytes. *Infect. Immun.,* **67**: 1050-1507.

Zurawski, G. and Dervies, J.E. (1994) Interleukin-13, an interleukin 4-like cytokine that acts on monocytes and B-cells, but not on T-cells. *Immunol. Today,* **15**: 19-26.